Universidade de Aveiro Departamento de Biologia
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Prof. Doutor Amadeu Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro e co-orientação do Doutor Reinier M. Mann, Investigador do Departamento de Ciências do Ambiente da Universidade de Tecnologia de Sidney (Austrália).

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À Beatriz e ao Bernardo

“Diz-me e eu esqueço,
Ensina-me e eu recordo,
Envolve-me e eu aprendo.”

Benjamin Franklin

o júri

presidente

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palavras-chave cádmio, biodisponibilidade, especiação de metais, transferência trófica, *Porcellio dilatatus,* Cd-cisteína, eficiência de assimilação, compartimentalização, sobrevivência, reprodução

resumo

Devido às actividades antropogénicas várias substâncias químicas têm sido introduzidas no meio ambiente em concentrações que de outro modo não ocorreriam de forma tão elevada naturalmente. Assim, o conhecimento acerca das características de um químico, tais como, o potencial para se acumular em diferentes níveis tróficos, a sua mobilidade dentro do ecossistema, a toxicidade específica e a bioacumulação, é fundamental para compreender os seus efeitos nos ecossistemas. Esta tese investiga a influência de especiação, na biodisponibilidade do cádmio (Cd) para o isópode *Porcellio dilatatus*, incluindo os efeitos de especiação do metal: (i) na assimilação do Cd, (ii) no modo como o Cd se distribui internamente no organismo, e (iii) como a sobrevivência e a reprodução são afectadas em isópodes terrestres. Num primeiro ensaio laboratorial avaliou-se a importância da transferência trófica na assimilação do Cd em *P. dilatatus*. Para tal analisou-se a eficiência de assimilação (EA) do Cd em isópodes, adicionado superficialmente ao alimento (alface) na forma de $Cd(NO_3)_2$ e contaminando o meio de crescimento da alface. A hipótese era de que a alface contaminada biologicamente através do cultivo em meio hidropónico contaminado teria uma maior proporção de complexos com proteína ou conjugado na forma de Cd (ex. Cd cisteína). A EA de Cd foi maior entre os isópodes que foram alimentados com o sal (71%, SE = 7%), do que entre os isópodes que se alimentaram de alface contaminada biologicamente (52%, SE = 5%), demonstrando-se assim num teste laboratorial que é provável que a especiação do Cd influencie a taxa de assimilação e acumulação do Cd. Na experiência alimentar que se seguiu, estudou-se em detalhe a especiação do metal comparando as EA do Cd conjugado com cisteína ($Cd(Cys)_2$) e na forma de $Cd(NO_3)_2$, com os quais se contaminou gelatina com alface. A utilização de Cd-cisteína, proporcionou uma forma experimental para explorar a biodisponibilidade do Cd complexado dentro do tecido biológico. Como esperado, a EA de Cd em isópodes alimentados com nitrato de Cd (64%, SE = 5%) foi maior do que no caso de isópodes alimentados com o conjugado de cisteína (20%, SE = 3%). De seguida estudou-se a distribuição subcelular das espécies de Cd assimilado através de um processo de fraccionamento. Supunha-se que as diferenças de especiação de Cd reflectiria diferentes estratégias de compartimentalização, com consequências ao nível da detoxificação, armazenamento celular e distribuição subcelular do metal. O "sequestro" na forma de metal biologicamente detoxificado (BDM = proteínas estáveis ao calor - HSP e grânulos ricos em metal - RMG) foi maior nos isópodes alimentados com $Cd(NO_3)_2$, sugerindo que são mais eficientes na detoxificação de Cd (22%) do que quando alimentados com $Cd(Cys)_2$ (15%). Foi também demonstrado que os isópodes alimentados com $Cd(Cys)_2$ possuíam níveis de armazenamento de Cd superior nas fracções sensíveis ao metal (MSF = organelos e proteínas desnaturadas pelo calor - HDP) consideradas fracções potencialmente vulneráveis e afectando os isópodes em termos de toxicidade. As diferentes distribuições internas que se seguiram à assimilação e detoxificação das diferentes espécies de Cd foram finalmente avaliadas em termos da sobrevivência e reprodução dos isópodes. O tratamento com $Cd(Cys)_2$ teve maior mortalidade, provavelmente devido à maior disponibilidade de Cd ingerido com implicações ao nível dos processos fisiológicos. Os isópodes alimentados com $Cd(NO_3)_2$ armazenaram o Cd nos MRG, como estratégia de detoxificação, sendo mais eficientes a detoxificar o Cd ainda que aumentando a concentração total do metal que se tornou menos tóxico para o isópode. Desta forma, o Cd nos grânulos não estava disponível para os processos fisiológicos e deixou de ser tóxico. Isso poderia estar relacionado com a resistência e tolerância aos metais devido à capacidade dos isópodes compartimentalizarem o Cd no hepatopâncreas, que actua como um mecanismo de detoxificação e contribui para a tolerância a altos níveis de cádmio. Em termos de parâmetros reprodutivos, observou-se uma redução de gestações e duração da gestação na presença de ambas as espécies de metal, mas no caso do $Cd(Cys)_2$ as gravidezes não se concluíram. O número de jovens produzido por fêmeas alimentadas com $Cd(NO_3)_2$ foi menor do que no controlo, mas os pesos dos juvenis foram superiores. Finalmente sugere-se assim que esta abordagem seja considerada em estudos do movimento trófico de metais nas cadeias alimentares dado que se espera que a especiação de metais implique diferentes fluxos, dentro de uma dada cadeia trófica.

keywords cadmium, bioavailability, metal speciation, trophic transfer, *Porcellio dilatatus*, Cd-cysteinate, assimilation efficiency, compartmentalization, survival, reproduction

abstract Human activities have introduced several chemicals into the environment that otherwise would not be found in such high concentrations in nature. Therefore the knowledge about the characteristics of a chemical, such as the potential to accumulate at different trophic levels, mobility within the ecosystem and specific toxicity and bioaccumulation in organisms needs to be achieved in order to understand its effects on the ecosystems. The present thesis investigates the influence of speciation, in the bioavailability of cadmium (Cd) to the isopod *Porcellio dilatatus*, including the effects of metal speciation in: (i) Cd assimilation, (ii) the way Cd distributes internally within the organism, and (iii) how survival and reproduction is affected in terrestrial isopods. In a first laboratory trial the importance of trophic transfer to Cd assimilation in *P. dilatatus* was evaluated. This was carried out by examining the assimilation efficiency (AE) of Cd in isopods provided with food (lettuce) superficially amended with $Cd(NO_3)_2$ and provided with lettuce grown in Cd-contaminated media. The hypothesis was that lettuce biologically contaminated via hydroponic culture in contaminated media would have a high proportion of Cd in the form of Cd-protein complexes or Cd-*S*-conjugates (e.g. Cd-cysteine). AE of Cd was greater among isopods that were fed the simple salt (71%, SE=7%), than among isopods feeding on biologically contaminated lettuce (52%, SE=5%), hence demonstrating that speciation of Cd is likely to influence the rate of Cd assimilation and accumulation in a laboratory test. In a following dietary experiment, metal speciation was further studied by comparing AE using Cd as Cd cysteinate ($Cd(Cys)_2$) and $Cd(NO_3)_2$ deployed in contaminated gelatines containing lettuce. The use of Cd-cysteinate provided an experimental device to explore the bioavailability of Cd that is complexed within biological tissue. As hypothesized the AE of Cd by isopods fed with Cd nitrate (64%, S.E.=5%) was higher than in the case of isopods fed with Cd-cysteine conjugate (20%, S.E.=3%). The subcellular distribution of the assimilated Cd species was then studied with a fractionating procedure. It was assumed that differences in Cd speciation would reflect different compartmentalization strategies with consequences at the manner by which metal was detoxified, stored in cells and distributed at subcellular level. Sequestration as biologically detoxified metal (BDM = heat stable proteins - HSP and metal-rich granules - MRG) was higher in isopods fed with $Cd(NO_3)_2$ suggesting that they are more efficient at detoxifying Cd (22%) than when fed with $Cd(Cys)_2$ (15%). It was also shown that isopods fed with $Cd(Cys)_2$ had a higher storage level of Cd in the metal-sensitive fractions (MSF = organelles and heat denatured proteins - HDP) being considered potentially vulnerable fractions affecting isopods in terms of toxicity. The different internal distributions that followed the assimilation and detoxification of different Cd species were finally evaluated as survival and reproduction in isopods. The $Cd(Cys)_2$ treatment had the highest mortality probably due to higher availability of the ingested Cd that impaired physiological processes. Isopods fed with $Cd(NO_3)_2$ stored Cd in the MRG as a detoxification strategy hence they were more efficient at detoxifying Cd which may had lead to increased metal body burdens although being less toxic to the isopod. In this way, Cd in granules was not available for the physiological processes and became non toxic. This could also be related to metal tolerance and resistance that may be attributed to the ability of isopods to compartmentalize Cd in the hepatopancreas, which acts as a detoxification mechanism and contributes to tolerance to high cadmium levels. In the presence of both metal species a reduction of pregnancies and pregnancy duration was observed in terms of reproductive endpoints but in the case of $Cd(Cys)_2$ all pregnancies were inconclusive. The number of juveniles delivered per female fed with $Cd(NO_3)_2$ contaminated food was lower than in the control but the juvenile weights were higher. In sum, it can be suggested that future studies examining the trophic movement of metals in food chains should consider this kind of approach where different flows within a trophic chain are expected depending on metal speciation.

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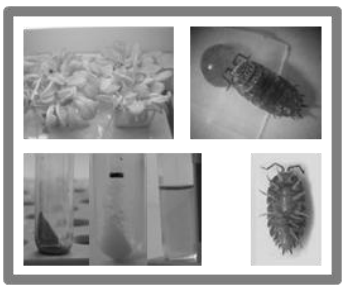
CHAPTER 1



Chapter 1. General Introduction and Objectives

1.1. Cadmium

Cadmium (Cd) is a nonessential metal considered a priority pollutant in Europe, hence being recently assessed for its risks to the environment and human health (ECB, 2007), as foreseen by the Council Regulation 793/93/EEC of March 1993 on the evaluation and control of risks of existing substances. Although Cd occurs naturally in soils and waters at low concentrations, deposition within the biosphere has increased dramatically over the last century as a consequence of anthropogenic activities. There are many uses for Cd that range from batteries, coating and alloys in the metallic form, to PVC heat stabilisers and pigments from the oxides. An important source of Cd is the fertilizers because Cd is present in the phosphate mineral rocks which are mined for use as raw material in the manufacture of phosphate fertilizers. Hence this metal is a multi-regulated substance in Europe. The most important piece of legislation is related to the restriction of the use of cadmium and cadmium compounds in products as defined in Annex XVII of Regulation (EC) 1907/2006 of December 2006 on the registration, evaluation, authorisation and restriction of chemicals (REACH), which has been directly applicable in all member states since 1 June 2009.

According to Council Directive 67/548/EEC of June 1967 on classification, packaging and labelling of dangerous substances Cd is considered as very toxic, carcinogenic, mutagenic and toxic to reproduction. Besides to its toxicity it has no known biological function (Odendaal and Reinecke, 1999). Effects to the environment have been mostly studied in the aquatic compartment and in the terrestrial compartment; most ecotoxicological tests have been performed with soluble Cd^{2+} salts (ECB, 2007). Standardized tests for soil fauna include the 14-day LC50 (50% lethal concentration) test using the earthworm *Eisenia fetida* (OECD, 1984) and the ISO test (ISO, 1999) with the collembolan *Folsomia candida*. Unlike many other toxic metals, Cd has the potential to bioaccumulate through soil-plant-animal food-chains (Nolan et al., 2003; McLaughlin et al., 2006; Mann et al., 2007). Bioaccumulation patterns among flora and fauna are dependent on both the environmental availability of Cd and physiological constraints on uptake into an organism, and both these aspects are in turn

dependent on its chemical speciation, i.e. the chemical form in which the metal is presented to the consumer. However other factors may affect metal uptake and accumulation such as the presence of other metals, pH, salinity, temperature, season, cation-exchange capacity of soils and the species taking up the Cd (Robards and Worsfold, 1991). Within a certain food chain there may exist biological compartments that posses tolerance and/ or detoxification mechanisms. As a consequence of these pathways, Cd may reach high concentrations in plants before phytotoxicity is manifested (Nolan et al., 2003), thereby providing a pool of Cd which may be available to herbivores. Looking at these primary consumers, tolerance to Cd depends on the ability of animals to regulate metal in many of their tissues and to accumulate excess metal in non-toxic forms in other particular tissues. The ecological consequence of such compartmentalization processes in prey organisms is that they may mediate the bioreduction or bioaccumulation of toxic metals along food chains, by altering metal bioavailability (Wallace and Lopez, 1997).

1.2. *Porcellio dilatatus*

Terrestrial isopods are invertebrates that play an important role in maintaining the structure and fertility of the soil. As saprophytic detritivores they are primary consumers alongside with millipedes and earthworms (Drobne, 1997; Loureiro et al., 2002). Invertebrate-mediated processes such as drainage, aeration, incorporation and degradation of organic matter are important in improving soil quality and energy flow through ecosystems (Drobne, 1997; Hornung *et al.*, 1998). Moreover, invertebrates are an important part of the terrestrial food web and constitute a significant component of the diet of other animals (Peijnenburg, 2002).

Isopods have been widely adopted as model species for the examination of metal accumulation and toxicity testing because of their extraordinary capacity to accumulate large body-burdens of toxic metals from the environment, predominantly in the hepatopancreas (Donker et al., 1990; Hopkin, 1990; Hames and Hopkin, 1991; Drobne, 1997; Hornung et al., 1998) with low to negligible depuration rates (Witzel, 2000). As hard-bodied soil invertebrates, the main route for accumulation of metals in terrestrial isopods is predominantly through dietary exposure rather than absorption through the body wall (Vijver et al., 2005). Isopods inhabit the upper layer of the soil and surface leaf litter, are quite abundant in southern Europe, being abundant in the field throughout the year, are

easily hand collected and are easy to handle under laboratory conditions, where they can complete their entire life-cycle (Caseiro *et al.*, 2000).

As saprophytic detritivores if the food they consume is contaminated with a metallic compound, only a limited proportion of that metal is likely to be present as free metal ions (Me^{+}). A large proportion of the metal is likely to be present in a form that has resulted from biological sequestration and transformation by either the micro-organisms growing on the decaying organic matter or by the organic matter itself while it was part of a living system (Ledin et al., 1999; Rauser, 1999; Magyarosy et al., 2002).

The woodlouse species *Porcellio dilatatus* Brandt (Crustacea, Isopoda) has been used in ecotoxicological experiments as it is an important representative of the invertebrate soil fauna and a valuable model for the examination of metal assimilation and accumulation. This woodlice is widely spread in Europe and tests performed aimed at studies of the effects to a wide range of toxicants, including metals (Mann *et al.*, 2005; Raessler *et al.*, 2005; Monteiro *et al.*, 2008) and organic compounds (Ribeiro *et al.*, 2001; Engenheiro *et al.*, 2005).

1.2.1. Putting reproduction of *P. dilatatus* into perspective

Terrestrial isopods allocate its available resources to growth, reproduction, or improving its survival, in a combination designed to maximize its fitness (Jones and Hopkin, 1996). *P. dilatatus* reproduces sexually and ovigerous females are considered iteroparous i.e. a single female is capable of breeding more than once in her lifetime (Achouri et al., 2008). Parental care is a behavioural strategy among many terrestrial isopods, and contributes to increased fitness of progeny. Eggs and juveniles (i.e. the manca stage) are carried by females in a marsupium that is provisioned with fluid from the mother and allows early development to take place independently of an external water source (Lardies et al., 2004). The mancae at emergence are relatively well advanced in age to cope with terrestrial life. The fact that gender and female pregnancy stage are easily distinguished, makes it easy to study the diverse aspects of their reproductive biology.

The most widely used toxicological endpoints in isopod testing are mortality (e.g. (Jansch *et al.*, 2005), growth and food consumption processes (e.g. (Loureiro et al., 2006), and reproduction (e.g. Vink and Kurniawati, 1996; Hornung *et al.*, 1997). The use of reproduction patterns as toxic responses is sometimes not convenient because of the long duration required for the test. Reproduction is also difficult to assess because after mating, females may retain the sperm for a long period of time before egg fertilization

(Vink and Kurniawati, 1996; Drobne, 1997). On the other hand using reproduction as a response endpoint to test sublethal effects of chemicals has the advantage of not killing the animals during the procedure, and at the end of the test they can be re-exposed to uncontaminated food in order to study recovery processes (Drobne and Strus, 1996). The effects of chemicals to reproduction traits are crucial to understand and transpose those effects to higher levels of organization. The impairment of reproductive processes are crucial for the population stability/growth and therefore isopods' role in decomposition processes and cycling of nutrients may be also affected.

1.3. Metal trophic transfer

The study of the trophic transfer of metals is a largely unexplored field. In recent years, a growing number of researchers have recognized the need to incorporate the principles of trophic transfer within the design of metal toxicity studies with invertebrates (e.g. Devi et al., 1996; Allinson et al., 2000; Merrington et al., 2001; Maryan'ski et al., 2002; Simon and Boudou, 2002; Green et al., 2003; Hendrickx et al., 2003; Wallace and Luoma, 2003; Hansen et al., 2004; Mann et al., 2004). These authors provided their test species with prey items that had accumulated metallic contaminants while still alive. In this way, they attempt to simulate the movement of metallic contaminants through the food chain, and thereby incorporate within their tests, the complexities of metal speciation and bioavailability in biological systems. Notwithstanding, the results of such studies are not easily predictable, because both the metal binding properties of the prey species and subsequent bioavailability to the predator are likely to be highly variable. For example, Harrison and Curtis (Harrison and Curtis, 1992) found the assimilation of Cd by trout to be much higher from biologically Cd-contaminated amphipods than Cd from an artificially contaminated diet. Conversely, lacewings feeding on aphids growing on Cd-contaminated media did not assimilate the Cd pool that had been accumulated by the aphids (Merrington et al., 2001). The dietary transfer of metals needs further investigation for both toxicological and regulatory standpoints (Nolan et al., 2003; Bechard et al., 2008).

1.3.1. Speciation and bioavailability

Chemical availability on the environment is related to the form in which the metal occurs and little is known about how metal speciation affects the way metals are absorbed, transported and stored in vivo and of how chelating agents can promote excretion of the

toxic metal (Cakir et al., 1999). Bioavailability has traditionally been defined to include the availability of metals to organisms as well as the availability of metals to tissues within organisms once inside the organisms. Although laboratory experiments, including biokinetic models, are useful in examining bioavailability, it is the behaviour of metals in ecosystems that is salient for ecological risk assessment (Peakall and Burger, 2003). The bioavailability of trace metals, their biological uptake, and their ecotoxicological effects on the soil biota can be better understood in terms of their chemical speciation. Assessment of the potential toxicity or bioaccumulation of metals by ecotoxicologists has increasingly used information on metal speciation to improve biological and biochemical toxicity models. Less progress has been made in terrestrial environments, mainly due to the difficulties in measuring metal activities in soil systems and also to the heterogeneous nature of the soil environment, where exposure of organisms to metals occurs through solid, liquid and gaseous pathways (Nolan et al., 2003). Consequently regulations or guidelines used to protect soil from metal pollution are still based on assessing the total concentration of metal present in the soil.

Bioavailability often determines whether the concentration at which a chemical is present will have effects on organisms. But the species with the highest concentration of metals are not necessarily the ones with the highest risk, because there are physiological mechanisms underlying the storage and excretion of metals (Peijnenburg and Jager, 2003). According to Vijver et al (2004), at cellular level, metals can be found in the following species: as free ionic form or complexed ion species (e.g. $CdCl_2$, $CdCl^+$, $CdCl^{3-}$); bound in the active centre of functional proteins and low molecular weight peptides; bound in the active centre of functional proteins and enzymes; bound to low molecular weight organic acids; bound to metallothionein, to transport proteins (e.g. ferritin), or other sequestration proteins; bound in vesicles of the lysossomal system, as intracellular granules; precipitated in extracellular granules, mineral deposits, residual bodies and exoskelotons; bound to cellular constituents potentially causing disfunction (e.g. DNA). These biochemical mechanisms serve to prevent the organism against accumulation of metal species, and they might also have an impact on the accumulation level reached in organisms during exposure. In the case of Cd exposure, the induction of metal-binding proteins such as phytochelatins and metallothioneins, as a mechanism of tolerance, plays an important role (Prasad, 1995). Phytochelatins and Metallothioneins (MTs) are small proteins with a significant concentration of cysteine (30%) (Ndayibagira et al., 2007), which contains a sulphydryl group and this fact accounts for the Cd-metallothionein

induction due to Cd high affinity for sulphur ligands (Zalups and Ahmad, 2003; Roosens et al., 2005).

1.3.1.1. FIAM and BLM

Some models have been developed in an attempt to link the bioavailability of contaminants and toxicity relying on the free ion metal activity (FIAM) or more recently on the metal binding with the proposed toxicological site of action (BLM) (Di Toro; 2001). The bioavailability of metals in soil is generally thought to be dictated by the free ion activity model (FIAM) which predicts that only metals existing as free Me^{+} are available for uptake across membranes (McLaughlin, 2002). The concentration of Me^{+} is dictated by physiochemical properties of the soil such as pH, the nature of metal exchange sites within the organic and inorganic matrices (McLaughlin et al., 2000; Peijnenburg, 2002), their binding affinity for soluble anionic ligands within soil pore-water (e.g. choride Lock and Janssen, 2003a; Weggler et al., 2004), and competition for those by sites with other cations in solution. These parameters dictate the “environmental availability” of a metal in any matrix. The biotic ligand model (BLM), developed for use with fish, expands on the FIAM by proposing the gill as a biotic ligand that competes with the various environmental exchange sites for Me^{+}-binding (Paquin et al., 2002). The difference between the BLM and FIAM is competitive binding at the biotic ligand, which models the protective effects of other metal cations, and the direct influence of pH (Nolan et al., 2003). The BLM considers not only the effect of the dissolved metal concentrations on toxicity, but also the metal interactions with organic and inorganic ligands—interactions that affect metal speciation and hence availability. Additionally, it incorporates the competitive interactions of metals and other cations with the organism at the site of action of toxicity, the biotic ligand (Peijnenburg and Jager, 2003). The capacity of the biotic ligand to bind and internalise metal ions (within the limitation of their environmental availability) is determined by physiological mechanisms and thereby dictates the “bioavailability” of metal ions. Bioavailability models like the BLM perform well with regard to predicting metal bioavailability in water-borne exposures (Niyogi and Wood, 2004), and is likely also to be predictive of metal bioavailability to plants and soft-bodied soil organisms where the major routes of exposure are absorption from pore-water directly across roots (Antunes et al., 2006) or body-walls (Peijnenburg, 2002; Lock and Janssen, 2003b). The digestive tract also acts as a biotic ligand (Hogstrand et al., 2002). However, the FIAM may not hold true with regard to the dietary exposure route because of the likely presence of active transport mechanisms that have the capacity to transport metal-bound organic (or inorganic)

complexes across the gut. Such mechanisms have been demonstrated in mammals (Sugawara and Sugawara, 1991; Groten et al., 1992) and trout (Harrison and Curtis, 1992a; Kjoss et al., 2006). Indeed, the studies in trout indicate that protein bound Cu or Cd is more readily taken up via the trout gut, than diets amended with simple metal salts. Absorption of metal complexes in the gut has also been demonstrated in aquatic crustaceans (for review see Fisher and Hook, 2002; Xu and Wang, 2002), however it remains unclear if the dietary form or speciation of the metal affects the assimilation efficiency.

1.3.1.2. Metal assimilation and assimilation efficiency

Determination of assimilation efficiency (AE) is an important endpoint when addressing contaminant bioavailability and very important parameter in understanding the trophic transfer and accumulation of a metal in animals from the ingested food. It is considered a first-order physiological parameter that can be quantitatively compared among different chemicals, species, and food particles under various environmental conditions, and has been defined as the fraction of ingested metal that is assimilated across the gut lining into the body tissue (Wang and Fisher, 1999). AE for metals has been shown to be directly proportional to metal bioaccumulation, which highlights the significance of AE in understanding and predicting metal bioaccumulation (Fisher et al., 1996).

1.4. Internal sequestration of metals in invertebrates

The internal distribution and detoxification of metals within an organism can be used to explain trophic transfer of metals but also to predict metal toxicity for the organism itself (Wallace and Lopez, 1996; 1997; Wallace and Luoma, 2003; Seebaugh *et al.*, 2006; Wang and Rainbow, 2006)

Just because a metal is available for uptake by an organism does not mean that it will be harmful because organisms are able to control metal concentrations in certain tissues of their body to minimize damage of toxic metals. The internal metal sequestration strategies of different species are complex and variable, and the determination of the metal concentrations in different compartments can be used to better understand mechanisms of accumulation and toxicity (Vijver et al., 2004). The way an organism makes his internal sequestration will directly influence trophic transfer to predators. The various internal metal fractions all have their own binding capacity for metals (Cheung et

al., 2007), which has implications for food-chain transfer to higher trophic levels. Metal compartmentalization is based on the different accumulation strategies organisms can follow after metal exposure and are indicators of toxicity. Once a metal is uptake by a consumer, physiological responses such as excretion from the metal excess pool and internal storage may occur in order to prevent adverse effects (Peijnenburg and Vijver, 2006). There is a need for partitioning the total body burden because only a portion of total body burden is biologically available for interaction with sites of toxic action.

1.4.1. Subcellular fractionation of Cd

A subcellular fractionation procedure (Wallace and Lopez, 1996; Wallace *et al.*, 2003; Wallace and Luoma, 2003) has been successfully applied in several studies of dietary accumulation of metals, particularly in marine food chains (Seebaugh and Wallace, 2004; Rainbow *et al.*, 2007; Steen Redeker *et al.*, 2007), with the purpose of explaining the variability observed in metal accumulation across the different species and food chains. This method has been considered dynamic in response to metal exposure and other environmental conditions, and takes into account metal- and organism-specificity (Wang and Rainbow, 2006). It is a simple and pragmatic approach in the prediction of trophic transfer of metals to higher trophic levels and is a first step for a practical tool that could explain most of the variability observed in metal accumulation and toxicity in organisms (Vijver et al., 2004). However, there is a need to further apply this approach in other food chains and other environmental compartments, such as terrestrial ecosystems, in order to verify its utility. Spiders *Dysdera crocata* fed with metal-contaminated isopods showed that not all metal fractions are equally available to higher trophic levels. Metals bound in granules of the hepatopancreas of woodlice were not absorbed by the predatory spider, but metals bound to ferritin (type C granules) were released and became available for uptake (Vijver et al., 2004).

In short, the procedure developed by Wallace and is co-workers (Wallace *et al.*, 2003; Wallace and Luoma, 2003) allows separating the accumulated metals associated with different subcellular compartments into five different fractions: cellular debris, granules, organelles, heat-denatured proteins, heat-stable proteins (MTs and PCs). The compartmentalization of metal with organelles and heat-denatured proteins “enzymes” could be viewed together as a subcellular compartment containing metal-sensitive fractions (MSF) related to toxicity; and heat-stable proteins (HSP) such as MT and metal-rich granules (MRG) as biologically detoxified metal (BDM) related to metal-detoxifying

capacity of an organism and potential tolerance (Wallace et al., 1998; Goto and Wallace, 2007), providing a more complete understanding of potential mechanisms of toxicity (Wallace et al., 2003).

The compartment MSF is considered vulnerable to metal exposure (i.e. non-specific binding) so the metals bound to organelles and HDP are considered metabolically available (Bechard et al., 2008). Wallace and Lopez (1997) showed that Cd associated with enzymes (HDP) and MT (HSP) in oligochaetes was absorbed by a grass shrimp with an efficiency of 100% and Cd associated in organelles with an efficiency of 70%. Wallace and Luoma (2003) first suggested that Cd associated with the subcellular fractions organelles, heat-denaturable proteins (HDP) and heat-stable proteins (HSP) in a bivalve were 100% assimilated by the grass shrimp suggesting that there is a trophically available metal compartment (TAM) for transfer to predator, while Cd bound to metal-rich granules was less bioavailable to predators. The significance of the subcellular distribution of accumulated metals in toxicity assessments is now receiving increasing attention among aquatic (e.g. Cheung *et al.*, 2006; Perceval *et al.*, 2006; Steen Redeker *et al.*, 2007) and terrestrial organisms (Vijver et al., 2006).

1.5. Objectives and thesis structure

The starting point of the present thesis was the FCT-funded project “TROPHA - Trophic assimilation of inorganic and organic pollutants in terrestrial invertebrates”, that aimed to address the question whether biologically contaminated diets confer greater bioavailability to environmental pollutants than superficially contaminated diets. This question had its foundation on a study from Harrison and Curtis (1992b) that showed that Cd accumulated by a live food source (amphipod) was assimilated more efficiently by rainbow trout than Cd added superficially to an artificial trout diet. The projected rationale stated that toxicity as a consequence of trophic transfer through the food chain remained a largely unexplored area of ecotoxicology, and in particular when soil ecotoxicology was concerned.

Hence a first laboratory trial had the purpose of evaluating the importance of trophic transfer to Cd assimilation in the terrestrial isopod *P. dilatatus*. This was carried out by examining the assimilation efficiency of Cd in isopods provided with food (lettuce) superficially amended with $Cd(NO_3)_2$ and provided with lettuce grown in Cd-contaminated media. The hypothesis was that lettuce biologically contaminated via hydroponic culture in

contaminated media would have a high proportion of the Cd in the form of Cd-protein complexes or Cd-*S*-conjugates (e.g. Cd-cysteine). In a following dietary experiment, metal speciation was further studied by comparing assimilation efficiencies using Cd as Cd cysteinate ($Cd(Cys)_2$) and $Cd(NO_3)_2$. The use of Cd-cysteinate provides an experimental device to explore the bioavailability of Cd that is complexed within biological tissue. Therefore Cd-cysteinate represents the most elementary form (species) of thiol-bound Cd in biological systems. The aim of such study was to detail the trophic movement of metals in the plant-isopod food chain. The distribution of the assimilated Cd was then studied with a fractionating procedure. Once again two species of Cd, as cysteinate and as nitrate, were provided in food. It was assumed that differences in Cd speciation would reflect different compartmentalization strategies with consequences at the manner by which metal was detoxified, stored in cells and distributed at subcellular level. The different internal distributions that followed the assimilation of different Cd species were finally evaluated as survival and reproduction in isopods.

In sum, the main objective of this dissertation was to test the hypothesis that speciation of metals influences bioavailability to the terrestrial isopod *P. dilatatus* and has consequences in the way metal distributes internally within the organism thus influencing Cd toxicity. Therefore, the following specific aims were pursued:

- Determination of cadmium AE from the diet, when Cd is presented as either a Cd-amended diet or pre-incorporated biologically into lettuce (*Lactuca sativa*), in the terrestrial isopod *P. dilatatus* – Chapter 2;
- Comparing the AE using Cd as cadmium cysteinate (Cys-S-Cd-S-Cys) (molar ratio Cd: Cys = 1:2) and also a Cd salt ($Cd(NO_3)_2$)., and hence the influence of Cd speciation on metal bioavailability to the terrestrial isopod, *P. dilatatus* – Chapter 3;
- Testing the hypothesis that different Cd species deployed in food – $Cd(Cys)_2$ and $Cd(NO_3)_2$ – would influence the manner by which this metal is detoxified, stored in cells and distributed at subcellular level, influencing the trophic transfer to the terrestrial isopod, *P. dilatatus* – Chapter 4;
- Determination of the toxicity effects of two species of Cd – $Cd(Cys)_2$ and $Cd(NO_3)_2$ – to survival and several reproductive parameters of the terrestrial isopod *P. dilatatus* – Chapter 5;

- Setting a general discussion and concluding remarks of this study – Chapter 6.

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CHAPTER 2

Chapter 2. Cadmium assimilation in the terrestrial isopod, *Porcellio dilatatus* – is trophic transfer important?

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Abstract

Terrestrial isopods have become important tools for the ecotoxicological assessment of metal-contaminated soils. Their value as an invertebrate model is partly because of their extraordinary capacity to bioaccumulate toxic metals from the environment. Replication of this accumulation process in the laboratory has in the past relied on the amendment of organic food substrates through the addition of simple metal salts. However, the bioavailability of the metals when presented through doping regimes, may differ from the bioavailability of metals in nature, because over time metals become biologically compartmentalised and complexed by organic molecules. This study examines the differential bioavailability of Cd to the terrestrial isopod, *Porcellio dilatatus*, when presented as either a Cd-doped diet or pre-incorporated biologically into lettuce *(Lactuca sativa*). Isopods were either provided with lettuce contaminated superficially with $Cd(NO_3)_2$ or lettuce grown hydroponically in growth media containing 100μM $Cd(NO_3)_2$. Assimilation efficiency of Cd was greater among isopods that were fed the simple salt (71%, SE=7%), than among isopods feeding on biologically contaminated lettuce (52%, SE=5%) and demonstrates that speciation of Cd is likely to influence the rate of Cd assimilation and accumulation in a laboratory test.

Keywords: Heavy metal, Cadmium, Bioavailability, Trophic transfer, Terrestrial ecotoxicology, *Lactuca sativa*.

2.1. Introduction

In recent years, a growing number of researchers have recognised the need to incorporate the principles of trophic transfer within the design of metal toxicity studies with invertebrates (Devi *et al.*, 1996; Allinson *et al.*, 2000; Merrington *et al.*, 2001; Maryanski *et al.*, 2002; Simon and Boudou, 2002; Green *et al.*, 2003; Hendrickx *et al.*, 2003; Wallace *et al.*, 2003; Hansen *et al.*, 2004; Mann *et al.*, 2004). All these authors provided their test species with prey items that had accumulated metallic contaminants while still alive. In this way, they attempted to simulate the movement of metallic contaminants through the food chain, and thereby incorporate within their tests the complexities of metal speciation and bioavailability in biological systems. The results of such studies are not easily predictable, because both the metal binding properties of the prey species and subsequent bioavailability to the predator are likely to be highly variable. For example, Hendrickx et al. (Hendrickx *et al.*, 2003) described extremely high levels of Cd assimilation and a complete lack of depuration of Cd in wolf spiders (*Pirata piraticus*) feeding on Cd-contaminated flies. Conversely, Hopkin and Martin (Hopkin and Martin, 1985) demonstrated that the spider, *Dysdera crocata* (a species that feeds exclusively on isopods), did not assimilate Cd or lead from contaminated isopods collected from a smelting works. In this case the difference appears to be related to the ability of *Dysdera crocata* to eliminate Cd prior to gut absorption and points to an evolutionary adaptation in a species that specialises in eating crustaceans known to bioaccumulate metals (Hopkin and Martin, 1985; Paoletti and Hassall, 1999).

The bioavailability of metals in soil is generally thought to be dictated by the free ion activity model (FIAM) which predicts that only metals existing as free metal ions (Me^{+}) are available for uptake across membranes (McLaughlin, 2002). The concentration of Me^{+} is dictated by physiochemical properties of the soil such as pH, the nature of metal exchange sites within the organic and inorganic matrices (McLaughlin *et al.*, 2000; Peijnenburg, 2002), their binding affinity for soluble anionic ligands within soil pore-water (Lock and Janssen, 2003; Weggler *et al.*, 2004), and competition for those by sites with other cations in solution. These parameters dictate the “environmental availability” of a metal in any matrix. The biotic ligand model (BLM), developed for use with fish, expands on the FIAM by proposing the gill as a biotic ligand that competes with the various environmental exchange sites for Me^{+}-binding (Paquin et al., 2002). The capacity of the biotic ligand to bind and internalise metal ions (within the limitation of their environmental availability) is determined by physiological mechanisms and thereby dictates the

"bioavailability" of metal ions. Bioavailability models like the BLM perform well with regard to predicting metal bioavailability in water-borne exposures (Niyogi and Wood, 2004), and is likely also to be predictive of metal bioavailability to plants and soft-bodied soil organisms where the major routes of exposure are absorption from pore-water directly across roots (Antunes et al., 2006) or body-walls (Peijnenburg, 2002; Lock and Janssen, 2003).

The digestive tract also acts as a biotic ligand (Hogstrand et al., 2002). However, the FIAM may not hold true with regard to the dietary exposure route because of the likely presence of active transport mechanisms that have the capacity to transport metal-bound organic (or inorganic) complexes across the gut. Such mechanisms have been demonstrated in mammals (Sugawara and Sugawara, 1991; Groten et al., 1992) and trout (Harrison and Curtis, 1992; Kjoss *et al.*, 2006). Indeed, the studies in trout indicate that protein bound Cu or Cd is more readily taken up via the trout gut, than diets amended with simple metal salts. Absorption of metal complexes in the gut has also been demonstrated in aquatic crustaceans (for review see Fisher and Hook, 2002; Xu and Wang, 2002), however it remains unclear if the dietary form or speciation of the metal affects the assimilation efficiency in invertebrates.

Because of their capacity to accumulate large body-burdens of toxic metals, terrestrial isopods have been widely adopted as model species for the examination of metal accumulation and toxicity testing (Drobne, 1997; Hornung et al., 1998a). Because terrestrial isopods are hard-bodied soil invertebrates, accumulation of Cd (among other metals) is predominantly through dietary exposure rather than absorption through the body wall (Vijver et al., 2005). It is also important to remember that isopods are saprophytic detritivores, and if the food they consume is contaminated with a metallic compound, only a limited proportion of that metal is likely to be present as free Me^{+}. A large proportion of the metal is likely to be present in a form that has resulted from biological sequestration and transformation by either the micro-organisms growing on the decaying organic matter or by the organic matter itself while it was part of a living system (Ledin et al., 1999; Rauser, 1999; Magyarosy et al., 2002). However, virtually all previous laboratory based examinations of metal accumulation and toxicity in terrestrial isopods have relied exclusively on addition of inorganic metal salts to organic substrates. The degree to which the metals in those studies were transformed into 'species' of greater or lesser bioavailability is dependent on the physiochemical environment and the degree of microbial activity within the experimental systems, and is therefore a source of variability within the experimental systems. The aim of this study was to examine the role of

biological metal sequestration in the assimilation efficiency of cadmium in a terrestrial isopod.

Cadmium was chosen because it is a priority pollutant in Europe (Council Directive 76/464/EEC), is readily accumulated by isopods with low to negligible depuration rates (Witzel, 2000), and permits comparisons with other animal models that have examined similar questions (Harrison and Curtis, 1992; Zalups and Ahmad, 2003; Mann *et al.*, 2006).

We provided terrestrial isopods with lettuce that had been, either:

1. Biologically contaminated via hydroponic culture in contaminated media. Lettuce contaminated in this way will have a high proportion of the Cd in the form of Cd-protein complexes or Cd-*S*-conjugates (e.g. Cd-glutathione, Cd-cysteine) (Maier et al., 2003), or
2. Superficially contaminated with $Cd(NO_3)_2$.

2.2. Materials and Methods

2.2.1. Food substrate

Lettuce was selected as a suitable food substrate on the basis of previous feeding and contamination trials (Mann et al., 2005). Three treatments (diets) were established to study the influence of metal speciation on the bioavailability of Cd to the terrestrial isopod *Porcellio dilatatus*.

1. Biologically contaminated lettuce (BCL)
2. Superficially contaminated lettuce (SCL)
3. Non-contaminated (control) lettuce (CON)

2.2.2. Test organisms

Isopods were selected from in-house cultures of *P. dilatatus* derived from individuals collected from a secondary coastal dune system in central Portugal. They were maintained at 20º C with a 16:8 h (light:dark) photoperiod on a substrate of sand within plastic containers. Alder leaves were provided as food (Caseiro et al., 2000; Kautz et al., 2000).

2.2.3. Lettuce growth and contamination

Lettuce (*Lactuca sativa* cv. Reine de Mai de Pleine Terre) plants were grown from seed as described in Mann et al. (2005). Briefly, lettuce seeds were germinated on a bed of perlite moistened with distilled water and subsequently grown hydroponically at 25 °C on a ~6 mm column of perlite within polystyrene seedling trays (24 mm; Polisur 2000, Huelva, Spain) floating on aerated nutrient media within plastic boxes. The nutrient media used for growth of lettuce was based on Hoagland's media: Macronutrients- KNO_3, 6 mM; $Ca(NO_3)_2$, 4 mM; $NH_4H_2PO_4$, 2 mM; $MgSO_4$, 2 mM; Micronutrients- H_3BO_3, 50 μM; $MnCl_2$, 10 μM; $ZnSO_4$, 0.77 μM; $CuSO_4$, 0.36 μM; Na_2MoO_4, 0.37 μM; Fe^{3+}-EDTA, 4.5 μM. For all plants a 16:8 h (light:dark) photoperiod was established with an array of fluorescent tubes (Mazdafluor Prestilflux TFP 36W/CFT) suspended ~30 cm above the seedlings/plants. After 5 weeks of culture the nutrient media was altered to include 100 μM Cd as $Cd(NO_3)_2$ (Mann et al., 2005). The Cd solution included 200 pCi ml^{-1} ^{109}Cd (PerkinElmer, Boston, MA, USA). The lettuce plants were grown within the contaminated media for a further 7 days with replacement of growth media every 2 days to avoid depletion of nutrients and changes in Cd concentration as a consequence of evaporation, exclusion from the plants or adsorption to plant roots (Mann et al., 2005). The plants were dried (2 days at 60 ºC) and individual leaves cut into sections (midvein excluded) according to desired mass (~10 mg) and Cd content. Cd content varied even within individual leaves (Mann et al., 2005). Therefore, leaf sections that contained between 300 and 600 μg Cd g^{-1} dry wt were selected for use in the experiment

Uncontaminated dried and sectioned lettuce designated for use as SCL was contaminated by topical addition of 10 μL $mg^{-1}$of a 360 μM $Cd(NO_3)_2$ stock solution that also contained 660 pCi ml^{-1} ^{109}Cd (PerkinElmer, Boston, MA, USA). The leaves were again dried before use. All leaf Cd-amended leaf sections were analysed for Cd by radiospectrometry to ensure that they contained approximately 400 μg Cd g^{-1} dry wt.

2.2.4. Feeding study

One day before starting the experiment a total of 60 juvenile isopods were selected by weight (mean = 47 mg, range = 35-65 mg) and isolated for 24 h without food to purge their gut. They were placed in individual polyethylene terephthalate (PET) boxes (∅ 85 mm x 43mm; Termoformagen, Leiria, Portugal). No distinction was made between sexes. The bottom of each box was replaced with a 2 mm nylon screen. Each of these boxes was inserted within a second box containing a thin layer of plaster of Paris mixed with

activated charcoal (8:1 vol:vol) for the retention of added moisture. The distance between the nylon screen and the plaster of Paris was ~5 mm. The screen allowed faecal pellets to drop through to the plaster substrate where they could be collected for weighing and analysis for Cd, and prevented coprophagy.

Twenty individuals were impartially allocated to each treatment. The food was cut into individual portions weighing between 5 and 10 mg (dry wt) and moistened before placing it within each box. Animals were fed for a period of 4 weeks exclusively on lettuce according to treatment. Faecal material was removed from the surface of the plaster of Paris every 2 days and dried (2 days at 60 ºC). At the end of each week, the food was replaced with fresh leaves of a known mass, and the remains of the old food were dried (2 days at 60 ºC) and weighed. The food was replaced to prevent the consumption of food which had become inoculated with fungi – the growth of fungi may have altered the bioavailability of Cd. At the end of 4 weeks, the isopods were left for 24 h without food to purge their guts, weighed and analysed for Cd by radio-spectrometry. Data on isopod, faecal pellet and leaf mass were used to determine indices of isopod growth, food consumption and assimilation efficiency.

2.2.5. Cadmium analysis

Sections of dry lettuce leaf (before feeding and lettuce remains after feeding), isopods and faecal matter were analyzed for Cd by radiospectometry. Samples were counted in a Genesis Gamma1 bench-top gamma counter (Laboratory Technologies, USA). Data on Cd content of leaves, isopods and faecal material were used to determine indices of Cd consumption and assimilation efficiency. The 360 μM and 100 μM contamination solutions were analysed for Cd by inductively coupled plasma spectroscopy (ICPS) in a Jobin Ivon JY70 with a Meinard C001 nebuliser. Specific activities of the 360 μM and 100 μM Cd contamination solution were assessed by comparing gamma counts with measurements obtained by ICP-MS.

2.2.6. Data analysis

Lettuce assimilation efficiency was calculated as:

$$AE_{lett}=(C_{lett}-F)/C_{lett}*100$$

where C_{lett} is mass of lettuce consumed, and F is the mass of faecal material produced. Cadmium assimilation efficiencies, were calculated as either:

$$AE_{Cd}=I_{Cd}/C_{Cd}*100$$

where I_{Cd} is the amount of Cd within the isopod at the termination of the feeding trial, and C_{Cd} is the amount of Cd consumed, or as:

$$AE_{Cd}=(C_{Cd}-F_{Cd})/C_{Cd}*100$$

where F_{Cd} is the amount of Cd within the faecal pellets.

SigmaStat (version 3.01, SPSS, Chicago, IL, USA) was used to perform all statistical tests. One-way ANOVA with Student-Newman-Keuls posthoc test was used to determine differences ($\alpha = 0.05$) in mass gain/loss, lettuce consumption, and lettuce assimilation efficiency among Controls, BCL and SCL treatment groups. Student *t*-tests were performed to determine differences ($\alpha = 0.05$) in indices of Cd consumption, assimilation and assimilation efficiency. Where data failed to fit a normal distribution, a Mann-Whitney rank sums test was employed ($\alpha = 0.05$).

2.3. Results

2.3.1. Analyses of Cd content in BCL and SCL treatment groups

ICP-MS analysis indicated that the nominally 360 and 100 μM contamination solutions were 365 and 102 μM respectively. Measured concentrations were used for all calculations. Biologically contaminated leaf sections provided to the BCL treatment group contained (mean ± SD) 391 ± 31 μg Cd g^{-1} dry wt (range: 338 to 450 μg Cd g^{-1} dry wt). Superficially amended leaf section provided to the SCL treatment group contained (mean ± SD) of 482 ± 94 μg Cd g^{-1} dry wt (range: 327 to 604 μg Cd g^{-1} dry wt).

2.3.2. Isopod growth, lettuce and Cd consumption, assimilation and assimilation efficiency

Isopods in all treatment groups lost weight during the trial (Figure 2.1A). Some animal mortality occurred in each treatment group – 5, 7 and 5 animals died in the CON, BCL and SCL treatment groups respectively.

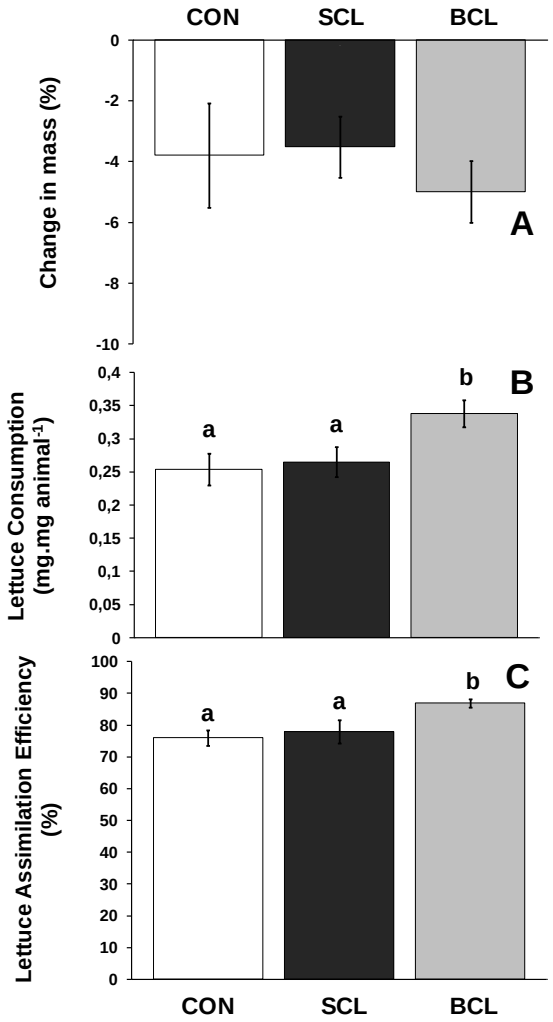

Figure 2.1. A. Change in isopod mass (%). B. Lettuce consumption by isopods. C. Lettuce assimilation efficiency by isopods. All error bars represent SE (n=13-15). Lower case letters a, b and c denote statistically significant (P < 0.05) groupings following an ANOVA with Student-Newman-Keuls post-hoc test. CON – Control lettuce; SCL – Superficial contaminated lettuce; BCL – Biological contaminated lettuce.

Lettuce consumption was low. Isopods ate approximately 0.3 mg mg animal (wet wt)$^{-1}$ over 4 weeks (Figure 2.1B). There were statistically significant differences in lettuce consumption between treatment groups (one-way ANOVA, P = 0.029; Student-Newman-Keuls, P< 0.05).

Lettuce assimilation efficiency (AE_{lett}) was high among all treatment groups (Figure 2.1C). There were statistically significant differences in AE_{lett} between biologically contaminated lettuce and control and between biologically contaminated lettuce and

artificially contaminated lettuce (one-way ANOVA, P = 0.020; Student-Newman-Keuls, P < 0.05).

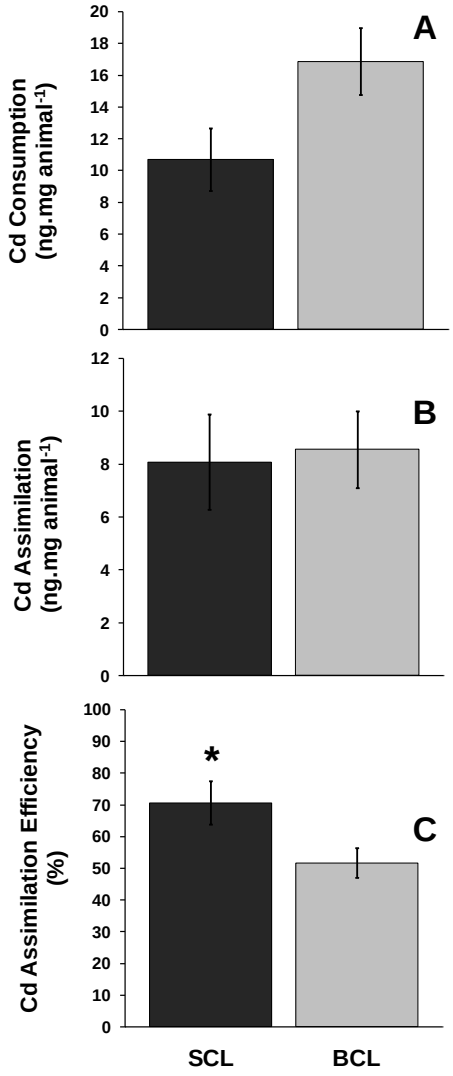

Figure 2.2. A. Cd consumption by isopods. B. Cd assimilation by isopods. C. Assimilation Efficiency of Cd by isopods. All error bars represent SE (n=14). The asterisk denotes a statistically significant difference (P < 0.05) following a student *t*-test. CON – Control lettuce; SCL – Superficial contaminated lettuce; BCL – Biological contaminated lettuce.

Although a *t*-test indicated a marginally insignificant difference (P = 0.054), isopods in the BCL treatment group consumed more Cd than the SCL groups (Figure 2.2A).

The amount of total Cd assimilated by the two treatment groups was the same (Figure 2.2B) with no statistically significant differences between them (Mann-Whitney, P

=0.240). Assimilation efficiencies, when calculated as $AE_{Cd}=I_{Cd}/C_{Cd}*100$ were (mean ± SE) 52 ± 5% and 71 ± 7% for the BCL and SCL group respectively (Figure 2C) with a statistically significant difference between them (t-test, P = 0.047). Assimilation efficiencies, when calculated as $AE_{Cd}=(C_{Cd}-F_{Cd})/C_{Cd}*100$ were (mean ± SE) 77 ± 2% and 84 ± 3% for the BCL and SCL group respectively (data not shown). The difference was not statistically significant (*t*-test, P = 0.089).

2.4. Discussion

This study provides support for the contention that Cd-speciation influences the level of Cd assimilation by terrestrial isopods. Isopods ate more lettuce if it had Cd biologically incorporated within it (BCL), and as a consequence they consumed more Cd than those isopods feeding on lettuce with Cd added superficially (SCL). Despite this, the actual amount of Cd assimilated by each treatment group was similar because the SCL group assimilated Cd more efficiently than those eating biologically contaminated lettuce (BCL). This result is consistent with results obtained in numerous studies with mammals (Zalups and Ahmad, 2003; Andersen *et al.*, 2004) and reptiles (Mann *et al.*, 2006) but contrary to those described for rainbow trout (Harrison and Curtis, 1992).

Consumption of lettuce was 3 to 4 times lower than observed in a previous study that indicated that lettuce was readily consumed and promoted growth in juvenile isopods (Mann et al., 2005). By contrast, isopods in this study lost weight irrespective of treatment group, with at least 25% mortality which is assumed to be related to inadequate nutrition. The only notable difference between the two studies was the age of the isopods. The earlier study used younger animals (~17 mg), and the imperative to eat is possibly greater among very young animals. Failure to eat at the commencement of this kind of test is a common problem when the food substrate is fresh leaf material. Leaves generally become more palatable to isopods only after the onset of microbial colonisation (Zimmer, 2002), possibly because microbial pre-conditioning tends to lower the C:N ratio (Zimmer et al., 2003). Many dietary studies have overcome this problem by either using alder leaves (Mann et al., 2005), which have inherently low C:N ratios (Kautz et al., 2000), or by augmenting leaf substrates with N rich pet-foods (e.g. Crommentuijn et al., 1995; Farkas et al., 1996; Hornung et al., 1998b; van Straalen et al., 2005). In this study, increased consumption would be expected if the leaves were left long enough to allow microbial preconditioning; however, it was important to avoid widespread microbial colonisation which might have changed the speciation of the Cd, particularly in the SCL treatment

group, so food could not be left for more than a week. Even during the course of one week, it is conceded that Cd bioavailability might change as a consequence chemical interactions within the moistened leaves.

More intriguing is the fact that there were higher levels of food consumption and assimilation among isopods in the BCL treatment group, than in either the controls or SCL treatment group. We can assume that the Cd itself did not bestow greater palatability upon the lettuce, because the SCL lettuce was no more (or less) palatable than the control food. However, it is possible that the contamination procedure itself altered the palatability of the lettuce. One of the *a priori* assumptions for these trials was that Cd incorporated biologically into the lettuce must exist predominantly as a Cd-*S*-conjugate or Cd-protein complex (Mann et al., 2005). This is a reasonable assumption because it is known that exposure to Cd^{2+} induces the production of amino acids, glutathione and cysteine rich phytochelatins in lettuce (Costa and Morel, 1994; Maier et al., 2003). Thus, an overall increase in N in the form of metal-binding organic content may afford the lettuce a greater degree of palatability to isopods.

Food assimilation efficiency was high (>70%) and has been a consistent characteristic of these studies when lettuce is provided as food. High food assimilation efficiency (>80%) was also described by Lirette et al. (1992) in snails provided with lettuce. Note however, that the high food assimilation rates in this study are likely to be an over-estimate because of the difficulty in accounting for all the faecal pellets produced by the isopods (see below).

The isopods in this study assimilated 52% (BCL) and 71% (SCL) of the Cd consumed, which is in stark contrast with assimilation rates found in vertebrates, which generally assimilate less than 10% of the Cd that enters the gut (Zalups and Ahmad, 2003; Mann *et al.*, 2006). The AE's reported in this study are also higher than those reported previously for isopods, which range from 30% up to 50% in *P. scaber* (Donker and Bogert, 1991; Khalil et al., 1995). However, Zidar et al. (2003) reported a range of AE's for Cd ranging from 32% to 100% for *P. scaber* feeding on hazel leaves augmented with 1000 μg Cd g^{-1} to 125 μg Cd g^{-1} respectively.

It could be argued that the higher rates of Cd assimilation in the SCL group could have resulted as a consequence of direct adsorption of Cd to the outer exoskeleton as isopods moved over the contaminated leaves, which is less likely to occur in the BCL group because the Cd is internalised within the lettuce. If this were the case, then an estimation of AE_{Cd} as $AE_{Cd}=(C_{Cd}-F_{Cd})/C_{Cd}$ is likely to provide an indication of gut AE_{Cd}

alone. AE_{Cd} calculated in this manner resulted in AE_{Cd}'s of 77% and 84% for the BCL and SCL treatment groups respectively. As expected these are over-estimations of AE_{Cd} because of the difficulty in accounting for all the faecal pellets produced by the isopods. Also, leaching of Cd from the faecal pellets to the plaster of Paris may have occurred (the plaster of Paris was not analysed for Cd). Accordingly, caution should be exercised in interpreting these data, which still indicate higher gut AE_{Cd} in the SCL group, but without a statistically significant difference between them. Perhaps more pertinent are findings of Vijver et al. (2005) who demonstrated that adsorption of Cd to the isopod exoskeleton does not occur, and that the Cd-burden is due exclusively to ingested Cd.

Numerous factors influence metal AE in terrestrial isopods. Zidar et al. (2003) demonstrated that food-metal concentration will effect AE of Zn, Cu and Cd in *P. scaber.* Abdel-Lateif et al. (1998) also performed a similar analysis of the influence of temperature and Cd concentration on the rate of Cd accumulation in *P. scaber* , while Hopkin (1990) demonstrated that different species have different accumulation capacities for Cd, Zn and Pb. Metal speciation can be added to the list of factors which will influence the level and rate of accumulation of metals.

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CHAPTER 3

Chapter 3. The influence of metal speciation on the bioavailability of cadmium to the terrestrial isopod, *Porcellio dilatatus*

Abstract

The ability of Cadmium (Cd) to bioaccumulate in biological organisms and possibly biomagnify through the food web makes it necessary to understand how speciation determines bioavailability, that in turn affects bioaccumulation patterns. Previous studies on plant-consumer Cd transfer drew attention to differences in Cd assimilation when it was pre-incorporated biologically into plant tissue, when compared to ionic Cd^{2+}, but the ecophysiological underpinning mechanism was not clarified. Cd is known for its high affinity for sulphur ligands in cysteine residues which form the basis for metal binding proteins such as metallothionein. This study compares Cd assimilation efficiency (AE) in *P. dilatatus* fed with cadmium-cysteine and $Cd(NO_3)_2$ in an examination of the influence of Cd speciation on metal bioavailability. As hypothesized, the AE of Cd by isopods fed with Cd nitrate (64%, S.E.=5%) was higher than in the case of isopods fed with Cd- cysteine conjugate (20%, S.E.=3%). This work demonstrates that the assimilation of Cd is greatly dependent on the chemical form of Cd presented to the isopod, i.e. its speciation.

Keywords: Cd-cysteinate, Trophic transfer, Assimilation efficiency, Dietary toxicity

3.1. Introduction

Cadmium (Cd) is a nonessential metal, considered a priority pollutant in Europe hence being recently assessed for its risks to the environment and human health (ECB, 2007) as foreseen by the Council Regulation 793/93/EEC of March 1993 on the evaluation and control of risks of existing substances. Although Cd occurs naturally in soils and waters at low concentrations, deposition within the biosphere has increased dramatically over the last century as a consequence of anthropogenic activities. Concern arises because unlike many other toxic metals, Cd has the potential to bioaccumulate through soil-plant-animal food-chains (McLaughlin et al., 2006; Mann et al., 2007). Bioaccumulation patterns among flora and fauna are dependent on both the environmental availability of Cd and physiological constraints on uptake into an organism, and both these aspects are in turn dependent on its chemical speciation, i.e. the chemical form in which the metal is presented to the consumer.

Metals that are distributed within the biosphere seldom occur as free metal ions. Free metal ions are highly reactive chemicals that have the capacity to disrupt biological systems. Therefore, when metal ions (even essential ions) enter biological organisms, numerous detoxification and sequestration pathways are initiated, to either deliver essential metals to the place where they are required or to isolate and eliminate toxic metals and prevent damage. Among vascular plants, mechanisms of tolerance include the induction of metal-binding proteins such as phytochelatins and metallothioneins (Prasad, 1995). Phytochelatins and Metallothioneins (MTs) are small proteins with a significant concentration of cysteine (30%) (Ndayibagira et al., 2007), which contains a sulphydryl group and this fact accounts for the Cd-metallothionein induction due to Cd high affinity for sulphur ligands (Zalups and Ahmad, 2003; Roosens et al., 2005). As a consequence of these detoxification pathways, Cd may reach high concentrations in plants before phytotoxicity is manifested (Nolan et al., 2003), thereby providing a pool of Cd which may be available to herbivores.

A previous dietary study, reported in the previous chapter, on the assimilation of Cd in the terrestrial isopod *Porcellio dilatatus* (Calhoa et al., 2006) indicated that the Cd speciation dictated the assimilation efficiency (AE) of Cd. Cadmium AE was higher among isopods provided with food (lettuce) superficially amended with $Cd(NO_3)_2$ than among isopods provided with lettuce grown in Cd-contaminated media. These results were consistent with the Free ion activity model (FIAM) that dictates that metals which are complexed with organic molecules are less bioavailable than free metal ions (Nolan et al.,

2003). Assuming a significant proportion of Cd that accumulates in lettuce is bound to sulphur ligands (Maier et al., 2003; Monteiro et al., 2008), we set out to specifically examine the bioavailability of Cd when bound to cysteine.

A dietary study was performed by feeding *P. dilatatus* with cadmium cysteinate (Cys-S-Cd-S-Cys) (molar ratio Cd: Cys = 1:2) and also a Cd salt ($Cd(NO_3)_2$). The aim of this study was to compare the AE using Cd as Cd cysteinate ($Cd(Cys)_2$) and $Cd(NO_3)_2$, and hence the influence of Cd speciation on metal bioavailability. This chapter assesses the advantages of using chemical speciation information to predict bioavailability of metals and its consequences to the environment on a trophic ecology approach.

3.2. Materials and Methods

3.2.1. Test organisms and culture conditions

Isopods were selected from laboratory cultures of *P. dilatatus* that have been maintained for more than 3 years and that were derived from individuals collected in a secondary coastal dune system in central Portugal. They were maintained on a substrate of sand in plastic containers at 20 ºC with a 16:8 h (light:dark) photoperiod. Alder leaves were provided *ad libidum* as a food source (Caseiro et al., 2000; Kautz et al., 2000) and distilled water was added to maintain moisture.

3.2.2. Lettuce and gelatine substrate

A mixture of lettuce leaves and gelatine was selected as a suitable food substrate to be used as the exposure route (Mann et al., 2005; Monteiro et al., 2008). The advantage of feeding isopods with gelatine discs is the reduced variability of Cd absorption among isopods within treatments (Wallace and Lopez, 1996). Also, because gelatine is derived from animal protein, its inclusion effectively decreased the C/N ratio (Kautz et al., 2000). Non-contaminated leaves of *Lactuca sativa* were reduced to powder using a mortar and pestle and were mixed with a gelatine solution prepared from 2.5 g gelatine powder (VWR Prolabo, Fontenay Sous Bois, France) and 12.5 ml deionised water (Milli-Q®) and then mixed by vortexing (Wallace and Lopez, 1996). Small portions of the mixture (gelatine discs) weighing approximately 6 µg (dry wt) were made and were pipetted onto Parafilm® (Pechiney Plastic Packaging, Menasha, WI, USA). These discs were stored frozen at -20 ºC until required (Wallace and Lopez, 1997).

Three treatments (diets) were established to evaluate the influence of metal speciation on the bioavailability of Cd to the terrestrial isopod *P. dilatatus*.

- $Cd(Cys)_2$ contaminated food- gelatine contaminated with Cd-cysteinate (including 462 µCi ml^{-1} ^{109}Cd as a tracer; Perkin-Elmer, Boston, MA, USA) mixed with non-contaminated leaves of *L. sativa*;
- $Cd(NO_3)_2$ contaminated food- gelatine contaminated with $Cd(NO_3)_2$ (including 23,1 µCi ml^{-1} ^{109}Cd as a tracer; Perkin-Elmer, Boston, MA, USA) mixed with non-contaminated leaves of *L. sativa*;
- Control food-gelatine mixed with non-contaminated leaves of *L. sativa*.

3.2.3. Cadmium-cysteine conjugate (Cys-S-Cd-S-Cys) (1:2)

Cadmium acetate (90 mM) including 462 µCi ml^{-1} ^{109}Cd (Perkin-Elmer, Boston, MA, USA) was added to L-cysteine (180 mM) in water while stirring. Sodium acetate (0.3 M) was added until a white amorphous precipitate formed. The precipitate was filtered off, washed with deionised water, and dried in the oven at 50 ºC (Barrie et al., 1993). This powder was kept at 4 ºC until required.

Cd content was analysed by inductively coupled plasma spectroscopy (ICPS) in a Jobin Ivon JY70 with a Meinard C001 nebuliser; confirming the molar ratio (1:2).

3.2.4. Feeding study

Before the start of the test, a total of 120 juvenile isopods were selected by weight (mean = 42 mg, ranging from 23 to 65 mg) and isolated individually in test boxes for 24 h without food to purge their gut. No distinction was made between sexes.

Polyethylene terephthalate (PET) test boxes were used (∅ 85 mm x 43 mm; Termoformagen, Leiria, Portugal) containing in the bottom a thin layer of plaster of Paris mixed with activated charcoal (8:1 v/v) for the retention of moisture.

Forty individuals were allocated to each treatment. Animals were fed for a period of 28 d exclusively on gelatine discs according to treatment. Gelatine discs that had been contaminated with $Cd(Cys)_2$ and $Cd(NO_3)_2$ were previously assayed for Cd by radiospectrometry [575 ± 118 and 296 ± 11 µg Cd/g dry wt (mean ± standard deviation, respectively)] before being fed to isopods. Gelatine discs were replaced every week to prevent the consumption of food which had become inoculated with fungi – the growth of

fungi may have altered the bioavailability of Cd. The remains of food were also weighed and Cd assayed by radiospectrometry. Faecal pellets were collected every 2 days to prevent coprophagy and dried (2 days at 60 ºC).

After 28 d, isopods were left for 24 h without food to purge their guts and subsequently weighed and analyzed for Cd burdens by radiospectrometry. Data on isopod, faecal pellet and gelatine mass were used to determine indices of isopod growth, food consumption and assimilation efficiency. The Cd content in isopods and in the gelatine discs were used to determine Cd assimilation efficiency (Cd AE).

3.2.5. Cadmium analysis

Dry gelatine discs (before and after feeding) and isopods were placed in 3.5-ml Röhren tubes (Sarstedt, Newtown, NC, USA) and were analyzed for Cd by radiospectrometry in a Genesis Gamma1 bench-top gamma counter (Laboratory Technologies, USA). The $Cd(NO_3)_2$ and $Cd(Cys)_2$ contamination solutions were analysed by inductively coupled plasma spectroscopy (ICPS) in a Jobin Ivon JY70 with a Meinard C001 nebuliser. Specific activities of the two contamination solutions were assessed by comparing gamma counts with measurements obtained by ICPS.

3.2.6. Data analysis

Food assimilation efficiency was calculated as:

$$AE_{food}=(C_{food} - F)/ C_{food} * 100$$

where C_{food} is the mass of gelatine discs (dry weight) consumed, and F is the mass of faecal material (dry weight) produced (Calhoa et al., 2006).

Radiospectrometry data obtained from the isopods and food were used to determine indices of Cd AE. Cadmium AE was calculated as:

$$AE_{Cd}=I_{Cd}/C_{Cd}*100$$

where I_{Cd} is the amount of Cd within the isopod at the end of the feeding trial, and C_{Cd} is the amount of Cd consumed.

All values presented in the Results section are mean values ± standard error.

SigmaStat (version 3.01, SPSS, Chicago, IL, USA) was used to perform all statistical tests. One-way analyses of variance (ANOVA) were performed to determine

differences (α=0.05) in changes in isopod mass, indices of food consumption and food assimilation efficiency among treatments. When necessary, data were transformed to achieve normality and equality of variance; when these criteria where not satisfied, the nonparametric Kruskal-Wallis one-way ANOVA was performed, followed by Dunn´s method post hoc test when differences were attained. Student´s *t*-tests were performed to determine differences (α=0.05) in indices of Cd consumption, assimilation and assimilation efficiency among treatment groups and a Mann-Whitney rank sums test was employed, when data failed to fit a normal distribution.

3.3. Results

3.3.1. Isopod growth, food consumption and assimilation efficiency

During the 28 d feeding experiment, only control isopods increased in weight (Fig. 3.1A). Growth among isopods provided with gelatine contaminated with $Cd(NO_3)_2$ was significantly lower than the control and $Cd(Cys)_2$ treatments ($P<0.05$). No significant difference was found in isopod growth when comparing control and $Cd(Cys)_2$ treatments. Mortality was below 10% in all treatments (4, 1 and 1 animals died in Control, $Cd(Cys)_2$ and $Cd(NO_3)_2$ respectively).

Isopods ate approximately 0.2-0.3 mg.mg animal^{-1} over the 28 d (Fig. 3.1B). Food consumption was significantly different between the $Cd(Cys)_2$ and the other two treatments ($P<0.001$). Feeding AE was significantly higher in isopods fed with $Cd(Cys)_2$ when compared with the other treatments ($p<0.05$). Isopods fed with control lettuce displayed AEs of 63 ± 4.3%, and treatments with $Cd(Cys)_2$ and $Cd(NO_3)_2$ food showed AEs of 81 ± 3.1% and 61 ± 4.3%, respectively (Fig. 3.1C).

3.3.2. Cadmium consumption, assimilation, and AE

Cd consumption (Fig. 3.2A) was significantly higher ($P<0.001$) in isopods fed with $Cd(Cys)_2$ gelatine although Cd assimilation was higher in isopods fed with $Cd(NO_3)_2$ gelatine (Fig. 3.2B). There were significant differences in Cd assimilation between treatments ($P=0.04$). The AE of Cd by isopods fed with $Cd(NO_3)_2$ gelatine (64.3 ± 4.47%) was higher than Cd AE of isopods fed with $Cd(Cys)_2$ gelatine (20.15 ± 2.82%) ($P<0.001$) (Fig. 3.2C).

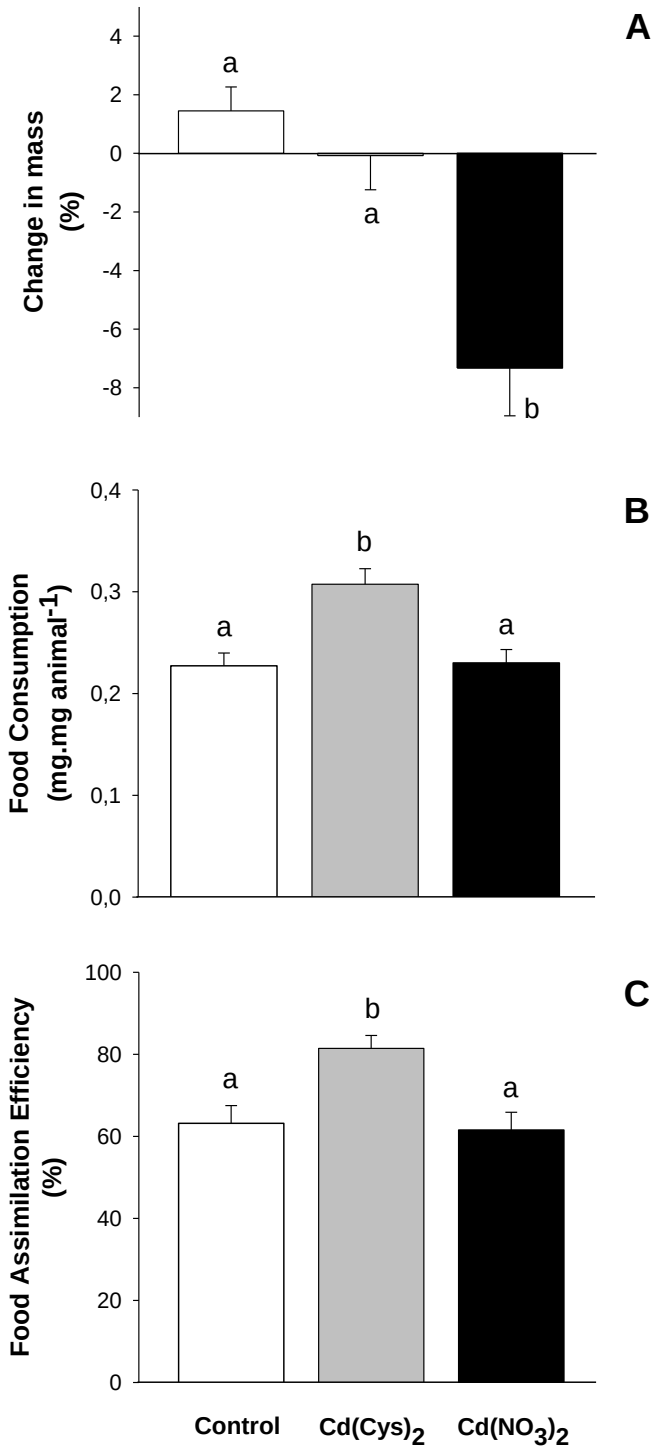

Figure 3.1. Food related traits of *Porcellio dilatatus* exposed to control, $Cd(Cys)_2$ and $Cd(NO_3)_2$ lettuce gelatines for 28 d. A. changes in isopod biomass (%); B. food consumption; C. food assimilation efficiency. All error bars represent SE (n=36-39). Lower case letters a, b and c denote significant differences among groups following an ANOVA with Dunn´s method post hoc test. Control - control food; $Cd(Cys)_2$ – $Cd(Cys)_2$ contaminated food; $Cd(NO_3)_2$ – $Cd(NO_3)_2$ contaminated food.

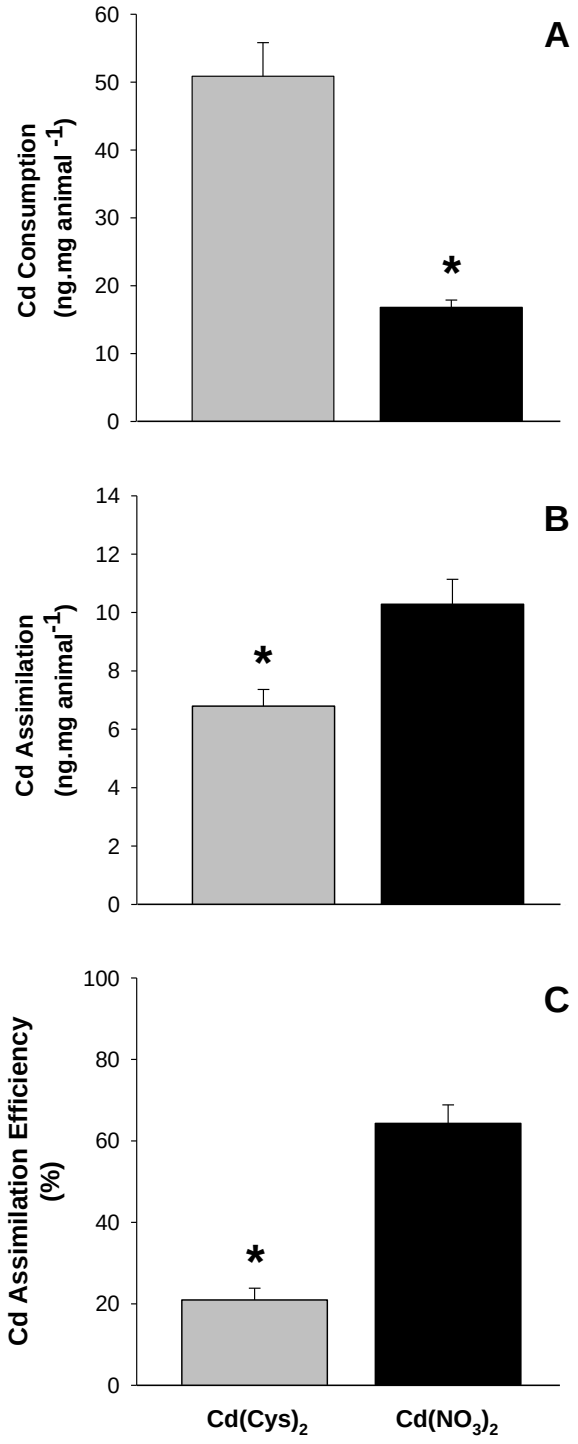

Figure 3.2. Cadmium related traits for *Porcellio dilatatus* exposed to control, $Cd(Cys)_2$ and $Cd(NO_3)_2$ lettuce gelatines for 28d. A. Cd consumption; B. Cd assimilation. C. Cd assimilation efficiency. All error bars represent SE (n=39). The asterisk denotes significant difference (student *t*-test; P<0.001). $Cd(NO_3)_2$ – $Cd(NO_3)_2$ contaminated food; $Cd(Cys)_2$ – $Cd(Cys)_2$ contaminated food.

3.4. Discussion

The use of Cd-cysteinate in this study provides an experimental device to explore the bioavailability of Cd that is complexed within biological tissues. Cysteine is the primary source of sulfydryl ligands in metal-binding proteins such as MTs or phytochelatins, and a proportion of the Cd that has been assimilated into biological tissues is likely to be associated with cysteine residues incorporated into MT or MT-like proteins (Monteiro et al., 2008). Therefore Cd-cysteinate represents the most elementary form (species) of thiol-bound Cd in biological systems.

The results in the present study are in accordance with findings from a previous study by Calhoa et al. (2006). In that study *P. dilatatus* were provided with lettuce contaminated superficially with either $Cd(NO_3)_2$ (SCL – superficially contaminated lettuce) or lettuce that had been grown hydroponically in Cd-contaminated media (BCL – biologically contaminated lettuce). That study relied on an assumption that a high proportion of Cd in the BCL treatment-group would be bound to Cd-S-conjugates. This assumption was born out by a subsequent sub-cellular fractionation study (Monteiro et al., 2008) that indicated that 22.4% of Cd was bound to the heat stable protein fraction (MT-like proteins). Therefore, in several aspects the present study parallels that of Calhôa et al (2006), with numerous similarities, but more importantly, some distinct differences, as follows.

Firstly, food consumption and assimilation indices are similar. As was the case with the BCL treatment group (Calhoa et al., 2006), food assimilation among isopods in the $Cd(Cys)_2$ treatment group was higher than other treatment groups, and likely reflects an increase in nitrogen (and subsequent increase in palatability) conferred by cysteine residues (Zimmer, 2002; Zimmer et al., 2003).

Secondly, in both studies growth was poor, although the inclusion of gelatine as a source of nitrogen in the present study did improve growth indices in control and $Cd(Cys)_2$ treatment groups. The negative growth rates among isopods in the $Cd(NO_3)_2$ treatment remain similar to that observed in Calhôa et al (Calhoa et al., 2006), and is therefore indicative of a distinct difference between the $Cd(NO_3)_2$ and $Cd(Cys)_2$ treatment groups. Growth inhibition among isopods is a commonly reported consequence of Cd exposure (Odendaal and Reinecke, 2004), and the fact that food assimilation in the $Cd(NO_3)_2$ treatment group was similar to controls indicates that the poor growth indices are not simply a reflection of an avoidance behaviour (Odendaal and Reinecke, 1999), but may reflect the increased cost of detoxification of Cd^{2+} (Zidar *et al.*, 2003). The absence of a

similar growth inhibition among isopods in the $Cd(Cys)_2$ treatment group is notable, and suggests that Cd, when presented as a Cd-S-conjugate, does not elicit the same metabolic response as Cd^{2+}. This inference requires verification because Cd assimilation was slightly (but significantly) lower in the $Cd(Cys)_2$ treatment group.

Isopods fed with $Cd(Cys)_2$ lettuce gelatine consumed more Cd than in the last experiment with the BCL treatment, as a consequence of higher gelatine Cd concentration. In the previous study the differences in the Cd assimilation were not so evident, being Cd assimilation for the BCL treatment a bit higher than in the SCL. One can also draw the hypothesis that higher rates of Cd assimilation as $Cd(NO_3)_2$ might have resulted as a consequence of direct adsorption of Cd from water vapour in the pleoventral space since isopods are in straight contact with contaminated food, although Vijver et al. (2005) demonstrated that the absorption of Cd is exclusively due to ingested Cd. Nonetheless Drobne (1993) demonstrated that in isopods water vapour absorption, cutaneous absorption and liquid water uptake take place by the mouthparts and/or uropods. These processes are based on active processes for liquid uptake which does not include penetration through the cuticle.

In the present study, the assimilation efficiency of $Cd(Cys)_2$ is relatively low (20%) compared to $Cd(NO_3)_2$, and much lower than the Cd-AE in the BCL group (~50%)(Calhoa et al., 2006), confirming the relatively low bioavailability of Cd associated with Cd-S-conjugates (Harrison and Curtis, 1992; Andersen et al., 2004; Mann et al., 2006; Monteiro et al., 2008). Monteiro et al. (2008) examined the subcellular distribution of Cd in lettuce following hydroponic contamination, and demonstrated that only a small proportion of metal (22.4%) was bound to a subcellular fraction (HSP) synonymous with phytochelatins or MT-like proteins. In the same study, Monteiro et al. (2008) provided isopods with isolated subcellular fractions, and similarly demonstrated that the Cd in the fraction containing Cd-S-conjugates (HSP) had a low AE (22.8%), which is close to the AE for Cd in $Cd(Cys)_2$ in the present study, suggesting that the isopods ability to assimilate $Cd(Cys)_2$ is the same as its ability to assimilate Cd-MT. Another interesting similarity with Monteiro et al. (2008) studies is that estimated Cd AE in all fractions in the isopod *P. dilatatus* fed with *L. sativa* were 44.1% similar to AE obtained on an previous study (Calhoa et al., 2006) for BCL of 52%.

Although bioavailability of Cd bound within Cd-S-conjugates has been demonstrated in the present study and elsewhere to be low, some Cd is assimilated, and it is possible that $Cd(Cys)_2$ and other Cd-S-conjugates are able to cross the gut epithelium (Groten et

al., 1991; Sugawara and Sugawara, 1991; Harrison and Curtis, 1992). Cd-cysteinate is known to behave as molecular mimics at the sites of specific transport proteins that normally serve to absorb aminoacids or oligopeptides (Zalups and Ahmad, 2003). As indicated above, the manner in which Cd-S-conjugates are metabolised or detoxified by organisms may be quite different to the manner in which they handle Cd^{2+}. The following chapters will examine the subcellular distribution of Cd when presented to isopods as different Cd species, and the potential difference in toxicity.

3.5. Conclusion

Results from this study clarify how the mechanisms by which plants sequester and detoxify accumulated metals can determine metal speciation and subsequent bioavailability to consumers. This chapter presents the direct assessment of the bioavailability of a Cd-cysteinate compared to a simple Cd salt ($Cd(NO_3)_2$), and demonstrated that Cd-speciation influences the level of Cd assimilation by terrestrial isopods. $Cd(Cys)_2$ was significantly less bioavailable than the Cd provided as $Cd(NO_3)_2$. Future studies that examine the trophic movement of metals in food chains should also consider this kind of approach, where different flows within a trophic chain are expected depending on metal speciation.

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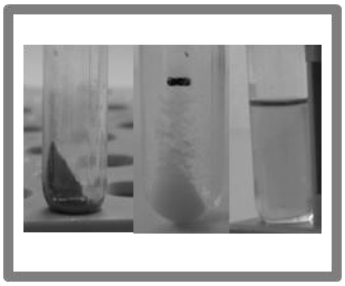
CHAPTER 4



Chapter 4. Does metal speciation dictate the subcellular distribution of cadmium in the terrestrial isopod *Porcellio dilatatus*?

Abstract

Cadmium (Cd) metal is present at potentially harmful concentrations in the environment but there is little information on its bioaccumulation and transfer up terrestrial food webs. Subcellular distribution of metal accumulated within an organism can be used to understand metal trophic transfer along a food chain and may provide valuable information about metal toxicity and tolerance. A previous dietary study with isopods showed that Cd cysteine conjugate decreased the assimilation efficiency of Cd when comparing with organisms fed with nutriment contaminated with the metal salt. The subcellular fractionation was adopted in this study as a tool to test the hypothesis that different Cd species deployed in food – $Cd(Cys)_2$ and $Cd(NO_3)_2$ – would influence the manner by which this metal is detoxified and stored in cells, thereby influencing the trophic transfer to isopods. Studied fractions were as follows: cellular debris, metal-rich granules (MRG), organelles, heat denatured proteins (HDP) and heat stable proteins (HSP). The organelles, HSP and HDP were considered trophically available fractions (TAM). Organelles and HSP were grouped as metal-sensitive fractions (MSF), and HDP and MRG were grouped as biologically detoxified metal (BDM). The cellular debris has the highest subcellular Cd distribution (59-64%) independently of the species of Cd. Sequestration as HSP and MRG (BDM) were higher in isopods fed with $Cd(NO_3)_2$ suggesting that they are more efficient at detoxifying Cd (22%) than when fed with $Cd(Cys)_2$ (15%). Thus our data suggests that estimates of TAM (trophically available metal) in isopods were not dependent on Cd species, that is available to a predator, being 24% in $Cd(Cys)_2$ and 22% in $Cd(NO_3)_2$.The results draw attention to the ecological relevance of the subcellular distribution of Cd in a consumer and highlights that a change in the speciation of Cd may have a direct impact in the Cd subcellular distribution which may affect the trophic transfer.

Keywords: Cd-cysteine, Dietary metal, Centrifugal fractionation, Trophic transfer.

4.1. Introduction

Metal availability on the environment is related to the form in which the metal occurs, and little is known about how metal speciation affects the way metals are absorbed, transported and stored *in vivo* and of how chelating agents can promote excretion of a toxic metal (Cakir et al., 1999). Hence it is important to consider the dietary route of metal exposure and the relevance of the complexity of internal metal subcellular partitioning in target organisms, which may significantly affect the subsequent transfer of metals to higher levels of the trophic chain (Wallace and Lopez, 1996b; 1997; Wallace and Luoma, 2003; Seebaugh *et al.*, 2006; Wang and Rainbow, 2006). The dietary transfer of metal needs further investigation for both toxicological and regulatory standpoints (Nolan et al., 2003; Bechard et al., 2008).

Some models have been developed in an attempt to link the bioavailability of contaminants and toxicity relying on the free ion metal activity (FIAM) or more recently on the metal binding with the proposed toxicological site of action (BLM) (Di Toro; 2001) but neither consider the complexity of internal metal subcellular fractionation, which may significantly affect metal toxicity and subsequent trophic transfer (Steen Redeker et al., 2007).

The internal metal sequestration strategies of different species are complex and variable. The way an organism makes its internal sequestration depends on different accumulation strategies that follow metal exposure. This can be explained by the fact that the various internal metal fractions have their own binding capacity for metals (Cheung et al., 2007), which has implications for food-chain transfer to higher trophic levels. Once a metal is uptaked by a consumer, physiological responses such as excretion from the metal excess pool and internal storage may occur, to prevent adverse effects (Peijnenburg and Vijver, 2006).

Subcellular partitioning of metals can provide valuable information about metal toxicity and tolerance (Wang and Rainbow, 2006), with the view of predicting metal toxicity for the organism itself, but it can also be used to explain trophic transfer of metals (Wallace et al., 2003). Subcellular fractionation (Wallace et al., 2003; Wallace and Luoma, 2003) has been successfully applied in several studies of dietary accumulation of metals, particularly in marine food chains (Wallace and Lopez, 1996b; Wallace et al., 2003; Wallace and Luoma, 2003; Seebaugh and Wallace, 2004; Zhang and Wang, 2006; Rainbow et al., 2007; Steen Redeker et al., 2007), with the purpose of explaining the variability observed in metal accumulation across different species and food chains. This

method has been considered dynamic in response to metal exposure and other environmental conditions, and takes into account metal- and organism-specificity (Wang and Rainbow, 2006). It is a simple and pragmatic approach in the prediction of trophic transfer of metals to higher trophic levels and is a first step for a practical tool that could explain most of the variability observed in metals accumulation and toxicity in organisms (Vijver et al., 2004).

Cysteine is present in metalothioneins and contains a sulphydryl group that accounts for the Cd-metallothionein induction due to Cd high affinity for sulphur ligands (Zalups and Ahmad, 2003; Roosens et al., 2005).

In a prelimary experiment, the effect of speciation in plant-isopod food chain was studied (Chapter 3) with a dietary trial that compared isopod uptake traits when feed with $Cd(Cys)_2$ and $Cd(NO_3)_2$. It was demonstrated that the cysteine conjugate decreased the assimilation efficiency of Cd when comparing with the isopods fed with nutriment contaminated with the metal salt.

A similar procedure of subcellular fractionation to the one developed by Wallace and co-workers (Wallace et al., 2003; Wallace and Luoma, 2003) was adopted in this study as a tool to explain the variability observed in Cd assimilation by isopods fed with different species of Cd. It is assumed that differences in Cd speciation reflect different internal compartmentalization strategies that will influence internal bioavailable concentration. Most previous studies on Cd in isopods have focused on their toxicity (Khalil et al., 1995; Odendaal and Reinecke, 1999) but there is few knowledge on how this metal is transferred along food chains. Here, we tested the hypothesis that different Cd species deployed in food – $Cd(Cys)_2$ and $Cd(NO_3)_2$ – would influence the manner by which this metal is detoxified, stored in cells and distributed at subcellular level, influencing the trophic transfer to the isopods.

4.2. Materials and Methods

4.2.1. Test organisms and culture conditions

Isopods were selected from laboratory cultures of *P. dilatatus* that have been maintained for more than 3 years and that were derived from individuals collected in a secondary coastal dune system in central Portugal. They were maintained on a substrate of sand in plastic containers at 20 ºC with a 16:8 h (light:dark) photoperiod. Alder leaves were

provided *ad libidum* as food source (Caseiro et al., 2000; Kautz et al., 2000) and distilled water was added to maintain moisture.

4.2.2. Lettuce and gelatine substrate

A mixture of lettuce (*Lactuca sativa* L.) leaves and gelatine was selected as a suitable food substrate to be used as the exposure route (Mann et al., 2005; Monteiro et al., 2008). The advantage of feeding isopods with gelatine discs is the reduced variability of Cd absorption among isopods within treatments (Wallace and Lopez, 1996b). Also, because gelatine is derived from animal protein, its inclusion effectively decreased the C/N ratio (Kautz et al., 2000). Non-contaminated leaves of *L. sativa* were reduced to powder using a mortar and pestle and were mixed with a gelatine solution prepared from 2.5 g gelatine powder (VWR Prolabo, Fontenay Sous Bois, France) and 12.5 ml deionised water (Milli-Q®), and then mixed by vortexing (Wallace and Lopez, 1996b). Small portions of the mixture (gelatine discs) weighing approximately 6 mg (dry wt) were made and were pipetted onto Parafilm® (Pechiney Plastic Packaging, Menasha, WI, USA). These discs were stored frozen at -20 ºC until required (Wallace and Lopez, 1997).

Two treatments (diets) were established to study the influence of metal speciation on the subcellular distribution of Cd to the terrestrial isopod *P. dilatatus*:

- $Cd(Cys)_2$ contaminated food- gelatine contaminated with Cd-cysteinate (including 462µCi ml^{-1} ^{109}Cd as a tracer; Perkin-Elmer, Boston, MA, USA) mixed with non-contaminated leaves of *L. sativa*;
- $Cd(NO_3)_2$ contaminated food- gelatine contaminated with $Cd(NO_3)_2$ (including 23,1µCi ml^{-1} ^{109}Cd as a tracer; Perkin-Elmer, Boston, MA, USA) mixed with non-contaminated leaves of *L. sativa*.

4.2.3. Feeding study

Before the start of the test, a total of 80 juvenile isopods were selected by weight (mean = 38 mg, ranging from 23 to 62 mg) and isolated individually in test boxes for 24 h without food to purge their gut. No distinction was made between sexes.

Polyethylene terephthalate test boxes were used (∅ 85 mm x 43 mm; Termoformagen, Leiria, Portugal) containing in the bottom a thin layer of plaster of Paris mixed with activated charcoal (8:1 v/v) for moisture maintenance.

Forty individuals were allocated to each treatment. Animals were fed for a period of 28 d exclusively on gelatine discs according to treatment. Gelatine discs that had been contaminated with $Cd(Cys)_2$ and $Cd(NO_3)_2$ were previously assayed for Cd by radiospectrometry [range: 575 ± 118 and 296 ± 11 µg Cd/g dry wt to (mean ± standard deviation respectively)] before being fed to isopods. Gelatine was replaced every week, to prevent food consumption already inoculated with fungi; this procedure avoids the changes in Cd bioavailability caused by fungi. Faecal pellets were collected every 2 days to prevent coprophagy.

After 28 d, isopods were left for 24 h without food to purge their guts and were weighed and analyzed for Cd burdens by radiospectrometry.

4.2.4. Subcellular Cd distribution in isopods

Differences in isopods subcellular Cd distribution were investigated using the methodology described by Wallace and co-workers (Wallace et al., 2003; Wallace and Luoma, 2003), with few modifications (Figure 4.1).

Replicates (n = 6) of 3 isopods each (weight range: 0.8 - 1.5mg) were homogenized in 2ml of Tris buffer at pH 7.6 (20mM; 1:10 (m/v) tissue to buffer ratio). The homogenate was centrifuged at 1450g for 15min at 4 ºC. The resulting pellet was re-suspended in 0.5ml distilled water and heated at 100 ºC for 2min. An equal volume of NaOH (1N) was then added followed by heating at 70 ºC for 1h. Afterwards it was centrifuged at 5000g for 10 min at 20 ºC. The pellet formed contained the metal-rich granules (MRGs) and the supernatant was designated cell debris, containing mainly cell walls, tissue fragments, and other cellular debris (Wallace et al., 1998). The supernatant of the first centrifugation step, containing the cytosol, was centrifuged at 100.000g for 1 h at 4 ºC to sediment organelle components (i.e., chloroplasts, mitochondria). The pellet was designated as the organelle fraction. The 100.000g supernatant containing the soluble fraction of the cytosol was then heat denatured at 80 ºC for 10min and cooled on ice for 10min. Heat-denatured proteins were separated from the heat stable proteins (HSPs) (MT-like proteins) by centrifugation at 50.000g for 10min at 4 ºC. All fractions were assayed for Cd by radiospectrometry and metal contents were used to calculate distributions of Cd within isopods based on summation of Cd content of the five subcellular fractions. Each fraction was subsequently assayed for radioactivity, and Cd content.

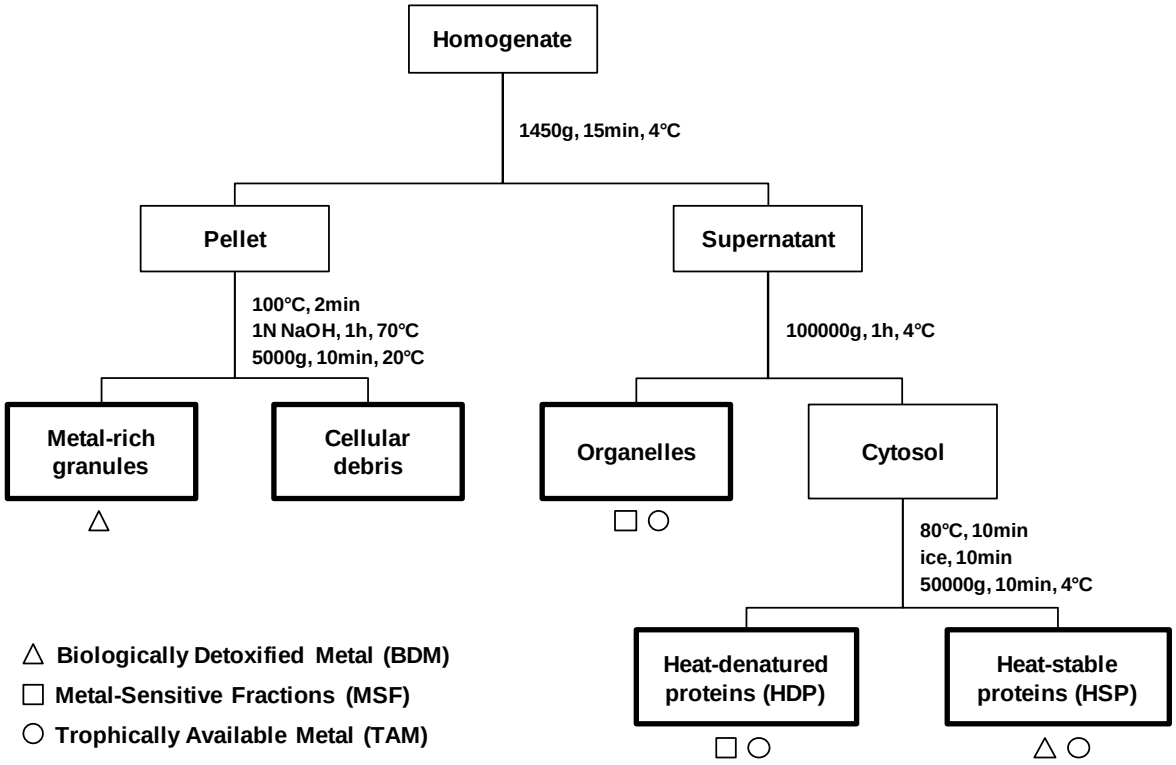


Figure 4.1. Procedure to obtain subcellular Cd distributions in isopods into five operationally defined fractions and three subcellular compartments with different biological significance (modified from Wallace and co-workers (Wallace et al., 2003; Wallace and Luoma, 2003)).

In total, five subcellular fractions were obtained: cellular debris (cell membranes and unbroken cells), metal-rich granules (MRG), organelles (mitochondria, microsomes, and lysosomes), heat denatured proteins (HDP) and heat stable proteins (HSP) like metallothionein (Wallace et al., 2003). The organelles, heat-denaturable proteins and metallothionein-like proteins were considered trophically available fractions (TAM) (Wallace and Luoma, 2003). Organelles and HSP were grouped as metal-sensitive fractions (MSF), and HDP and MRG were grouped as biologically detoxified metal (BDM) (Wallace et al., 2003) (Figure 4.1).

4.2.5. Cadmium analysis

All isopods and subcellular fractions were placed in 3.5-ml Röhren tubes (Sarstedt, Newtown, NC, USA) and were analyzed for Cd content by radiospectrometry in a Genesis Gamma1 bench-top gamma counter (Laboratory Technologies, USA). The $Cd(NO_3)_2$ and $Cd(Cys)_2$ contamination solutions were analysed for Cd by inductively coupled plasma atomic emission spectroscopy (ICP-AES; Jobin Ivon JY70 with a Meinard C001 nebuliser). Specific activities of the two contamination solutions were assessed by comparing gamma counts with measurements obtained by ICP-AES.

4.2.6. Statistical analysis

Cd assimilation and fractionation by isopods was analysed using a two-way ANOVA – with interaction between factors (i) Cd species, i.e. Cd-cys and $Cd(NO_3)_2$, (ii) and fractions (Cellular debris, MRG, Organelles, HSP and HDP) and compartments (BDM, MSF and TAM) – with the SigmaStat (version 3.01, SPSS, Chicago, IL, USA) and pair-wise multiple comparison procedures with the Student-Newman-Keuls method whenever significant differences between treatments were found. Statistical analysis was carried out for a significance level of 0.05. Data on the percentage compartmentalization were arcsine transformed to ensure normality and homoscedascity of data.

4.3. Results

To evaluate the distribution among body compartments, two levels of biological organization were considered in this study: the whole individual and the subcellular fractions following feeding experiment where $Cd(Cys)_2$ has 20% of AE of Cd and $Cd(NO_3)_2$ has 64% *Porcellio dilatatus* (Chapter 3).

Comparing subcellular fractionation of Cd between $Cd(Cys)_2$ and $Cd(NO_3)_2$ treatments in relation to the total assimilated Cd revealed significant differences ($F_{4,50}$ = 9.337; $p<0.001$) (Figure 4.2). In fact there is a statistically significant interaction between Cd speciation and respective fractionation according to the two-way ANOVA calculations ($p<0.001$). The cell debris fraction is significantly different from the other fractions ($p<0.05$) and represents the biggest storage of the total accumulated Cd (59-64%) in both treatments used. Within the cells of *P. dilatatus*, storage of Cd (Figure 2) for i) $Cd(Cys)_2$ was located in: cellular debris (64%)> HDP (12.1%) > MRG (11.8%) > organelles (9.2%) >

HSP (2.9%); and for ii) $Cd(NO_3)_2$ located in: cellular debris (59,1%) > MRG (19%) > HDP (12,5%) > organelles (6,2%) > HSP (3,3%).

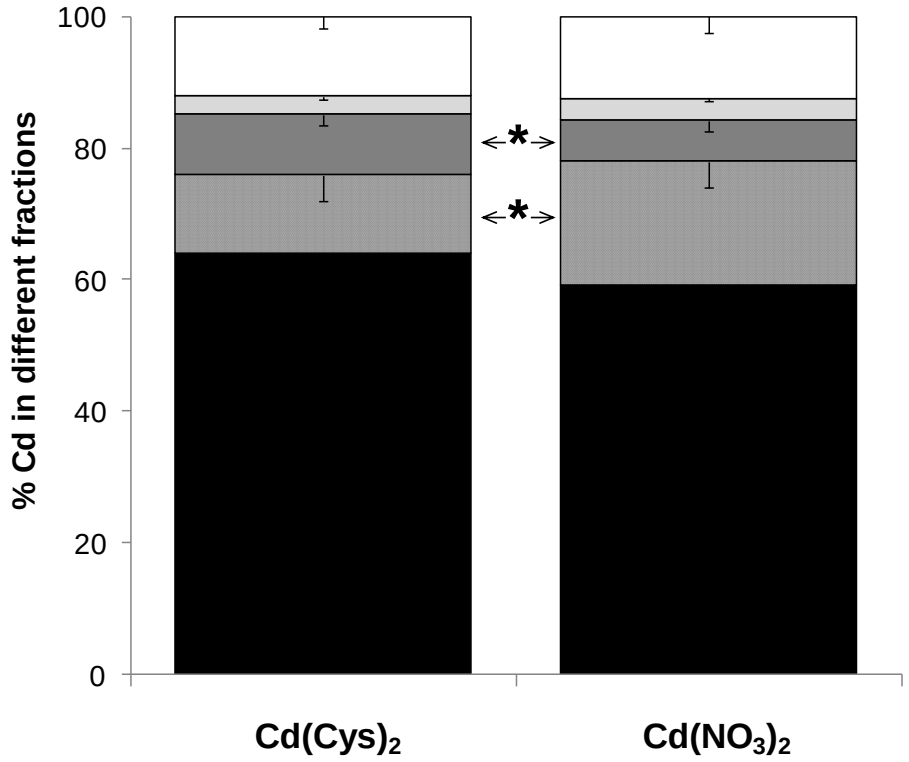


Figure 4.2. Percentage distribution of Cd in subcellular fractions of *Porcellio dilatatus* (means ±SD, n=6) fed $Cd(Cys)_2$ or $Cd(NO_3)_2$. Significant differences between $Cd(Cys)_2$ and $Cd(NO_3)_2$ fractions (P≤0.05) are indicated with asterisk.

Considering Cd speciation as one of the factors for the two-way ANOVA, no significant differences were found between $Cd(Cys)_2$ and $Cd(NO_3)_2$ treatments ($F_{2,30}$ = 9.954; p=0.356) although the analysis revealed that there is a significant interaction between Cd speciation and metal compartmentalization (p<0.001). In fact, BDM compartment is significantly different (p<0.05) between treatments. Isopods fed with $Cd(NO_3)_2$ stored more Cd in the BDM compartment (Figure 4.3).

Cadmium that is theoretically available for transfer to higher trophic levels, i.e. TAM: HSP + HDP + Organelles (Wallace and Luoma, 2003), represent 24% and 22% of the total assimilated Cd for isopods treated with $Cd(Cys)_2$ and $Cd(NO_3)_2$, respectively.

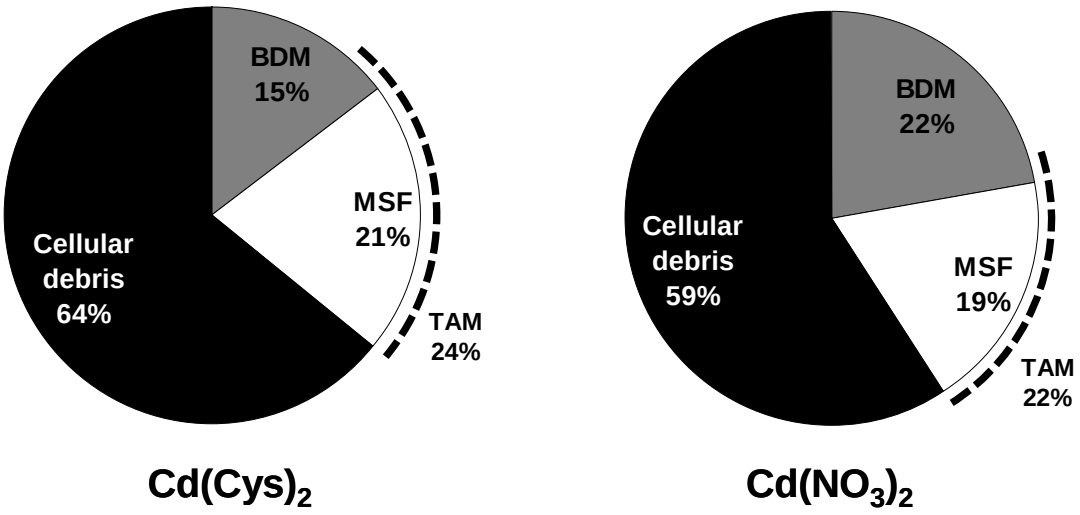


Figure 4.3. Subcellular compartments in *Porcellio dilatatus* fed with $Cd(Cys)_2$ and $Cd(NO_3)_2$ based on the biological significance of the various subcellular fractions. MSF = Organelles + HDP; BDM = HSP + MRG, Cellular debris and TAM (arc) = HSP + HDP + MRG.

Within each Cd treatment, only in the case of $Cd(Cys)_2$ significant differences were found between compartments, namely between BDM and the other two compartments.

4.4. Discussion

This study evaluated the subcellular distributions of Cd in isopods fed with $Cd(Cys)_2$ and $Cd(NO_3)_2$. Previous work (Chapters 2 and 3) had shown that these two species of Cd differed in terms of their assimilation efficiency to isopods and in terms of bioavailability.

Examining whole metal body burdens may not always be sufficient to explain ecotoxicological significance in terms of detoxification, toxicity and trophic transfer, being important the analysis of internal distribution of metals within an organism also in order to predict metal toxicity for the organism itself. Concentration of Cd in subcellular fractions varies depending on the specific organ (Perceval et al., 2006), species (Zhang and Wang, 2006), size of the body, as well as with the season (Wallace et al., 2003). The compartmentalization of metal as a subcellular compartment containing metal-sensitive fractions (MSF) is related to toxicity and is the metal considered metabolically available (Bechard et al., 2008). The biologically detoxified metal (BDM) compartment is related to

metal-detoxifying capacity of an organism and potential tolerance (Wallace et al., 1998; Goto and Wallace, 2007), providing a more complete understanding of potential mechanisms of toxicity (Wallace et al., 2003). Wallace and Lopez (1997) showed that Cd associated with enzymes (HDP) and MT (HSP) in oligochaetes was absorbed by a grass shrimp with an efficiency of 100% and that Cd associated in organelles was absorbed with an efficiency of 70%. Wallace and Luoma (2003) first suggested that Cd associated with the subcellular fractions organelles, heat-denaturable proteins (HDP) and heat-stable proteins (HSP) in a bivalve were 100% assimilated by the grass shrimp, indicating these fractions as a trophically available metal compartment (TAM) for transfer to predators.

The cellular debris is the only subcellular fraction that was not included in the compartmental analysis and includes tissue fragments, cell membranes and other cellular components of unknown consequence in terms of function. The present work reveals that the cellular debris has the highest subcellular Cd distribution (59-64%) independently of the species of Cd, which is in good agreement with results obtained by Monteiro et al. (2008) for Cd subcellular distribution of *L. sativa* deployed to the isopod *P. dilatatus*. The authors found 43.8% of accumulated Cd in the cell debris fraction in *L. sativa*. Inouye et al. (2007) reported that 85% of lead in the exoskeleton fraction of isopods was not available to predators so there is a great probability that the majority of the total Cd body burden in isopods may not be available to predator species because the highest Cd concentration were found in the cellular debris.

On cellular sequestration there are two major strategies of detoxification. One involves the binding of metals to heat-stable proteins (HSP) and the second one involves the formation of metal-rich granules (MRG). The role of MT in Cd binding (i.e. percentage of Cd bound to HSP) compared to the other subcellular fractions appears to be lower in this study when compared to other investigations with organisms exposed to Cd via food (Giguere et al., 2006; Monteiro et al., 2008). In both tested species of Cd, the HSP fraction does not appear to play an important role in detoxification processes for Cd, as expected (2,9% in $Cd(Cys)_2$ and 3,3% in $Cd(NO_3)_2$). The second detoxification strategy in invertebrates is metal storage in MRG. *P. dilatatus* fed with $Cd(NO_3)_2$ accounted for 19% in MRG, whereas only 11,8% of the total Cd was found in MRG of *P. dilatatus* fed with $Cd(Cys)_2$. MRG has been found in intestinal cells of many invertebrates (Vijver et al., 2006), including isopods (Dallinger and Prosi, 1988), where is known that Cd that enters in the hepatopancreas is mainly present in the S (small) cells, which consist of granules.

If sequestration as HSP and MRG (BDM) is considered a mode of detoxification, we can suggest that isopods fed with $Cd(NO_3)_2$ are more efficient at detoxifying Cd (22%) than when fed Cd(Cys) (15%), which can lead to increased metal body burdens although being less toxic to the isopod. This could be also related to metal tolerance and resistance, being such subcellular compartmentalization approach important to interpret differences in toxicity. During our 28d study, storage to MRG and HSP were used as mechanisms of detoxification of both Cd species, however Wallace et al. (2003) showed that during long periods of extreme exposure, these mechanisms may become overwhelmed and metals may bind to more sensitive cellular components, such as organelles and HDP (MSF), resulting in toxicity. In fact, it was observed a higher storage level in the MSF compartment in $Cd(Cys)_2$ treatment, although not significant in comparison to $Cd(NO_3)_2$ treatment, showing that Cd in organelles and in HDP are potentially vulnerable fractions to both metal exposures, and tend to be more sensitive to $Cd(Cys)_2$ treatment and consequently causing higher toxicity in isopods.

Wallace et al. (1998) showed that Cd-resistant worms produced both MT and MRG for Cd storage and detoxification while non-resistant worms only produced MT in response to Cd. Differences in subcellular distribution of Cd between resistant and nonresistant worms directly affected Cd availability for a predatory shrimp. There is no evidence that the same is true for isopods because to our knowledge no previous study investigated the subcellular partitioning of Cd within terrestrial isopods used as prey, but our data suggests that MRG might play a more relevant role in tolerance to long-term exposure than MT, that might act to protect against short-term Cd exposure.

TAM has been defined as an additional compartment because the subcellular partitioning of metal within prey may be related to metal trophic transfer (Wallace and Lopez, 1996a; 1997; Wallace *et al.*, 2003). In several studies linear relationships were found between TAM and assimilation by predators (Wallace and Luoma, 2003; Seebaugh and Wallace, 2004; Cheung and Wang, 2005; Seebaugh et al., 2006; Zhang and Wang, 2006). However, it is common to find cases where TAM does not show a strong relationship to trophic transfer, as reported by Seebaugh et al. (2005) where 68% of Cd was trophically available in grass shrimp but only 3-19% Cd was assimilated by the predator fish. Similarly, Steen Redeker et al. (2007) reported 72% trophically available Cd in *Tubifex tubifex* but only 9.8% was assimilated by the predator carp. Furthermore, there are several studies which indicate that not only trophically available fractions were available to the predator but also insoluble fractions (such as MRG) could be bioavailable to the predator (Cheung and Wang, 2005; Zhang and Wang, 2006). In our study, if

sequestration as HSP, HDP and MRG are considered as a component of TAM, we can conclude that both species of Cd used to feed isopods have similar (TAM in $Cd(Cys)_2$= 24%; TAM in $Cd(NO_3)_2$ = 22%) accumulated Cd available to predators. Thus our data suggests that estimates of TAM in isopods were not dependent on Cd species.

In sum, this study shows that total tissue burdens in prey may not be directly related to metal transfer to predators and the subcellular partitioning results are more useful when individual fractions were grouped into compartments MSF and BDM, demonstrating that the variability observed in metal partitioning can be useful in explaining toxicity. Moreover, herewith it is demonstrated that the subcellular distribution of Cd in isopods can be modified by metal speciation and subcellular fractionation of metal-binding in tissues, clarifying the mechanisms for metal toxicity and how the organisms detoxify metals. The results highlight that a change in the speciation of Cd may have a direct impact in the Cd subcellular distribution, affecting the trophic transfer.

Acknowledgments

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CHAPTER 5

Chapter 5. Survival and reproductive traits in *Porcellio dilatatus* exposed to different Cd species

Abstract

The woodlouse *Porcellio dilatatus* (Crustacea) is a suitable model species for the examination of toxic effects following metal assimilation and accumulation. In this study, the influence of cadmium speciation in survival and reproduction of isopods was investigated. Survival, growth and reproductive parameters (time to pregnancy, pregnancy duration, pregnancy and abortion occurrence, number of juveniles per female and juvenile weight) were recorded when isopods were exposed to two species of Cd deployed in food: $Cd(Cys)_2$ or $Cd(NO_3)_2$. There was a difference between survival rates of $Cd(Cys)_2$ exposed males and females and an higher acute toxicity was also observed when compared to males exposed to $Cd(NO_3)_2$. In the presence of both metal species a reduction of pregnancies and pregnancy duration was observed, but in the case of $Cd(Cys)_2$ all pregnancies were inconclusive. The number of juveniles delivered per female fed with $Cd(NO_3)_2$ contaminated food was lower than in the control but the juvenile weights were higher. As far as we are aware, the present study is the first demonstrating that metal speciation affects reproduction. $Cd(Cys)_2$ showed to be more toxic in this long term exposure and to jeopardize completely the reproduction effort of isopods.

Keywords: Cadmium-cysteine, Reproduction, Survival, Isopods

5.1. Introduction

Terrestrial isopods are saprophytic detritivores that inhabit the upper layer of the soil and surface leaf litter, and that play an important role in maintaining the structure and fertility of soils (Drobne, 1997; Loureiro et al., 2006). Invertebrate-mediated processes such as drainage, aeration, incorporation and degradation of organic matter are important in improving soil quality and energy flow through ecosystems (Drobne, 1997; Hornung et al., 1998b; Odendaal and Reinecke, 1999).

The woodlouse species *Porcellio dilatatus* (Crustacea) has been often chosen as a model species as it is an important representative of the invertebrate soil fauna (Sousa et al., 1998; Caseiro et al., 2000; Ribeiro et al., 2001; Engenheiro et al., 2005; Calhoa et al., 2006). Terrestrial isopods are also easily cultured under laboratory conditions, where they can complete their entire life-cycle (Caseiro et al., 2000). Moreover they have been widely used for the examination of metal accumulation and toxicity testing because of their extraordinary capacity to accumulate large body-burdens of toxic metals from the environment, predominantly in the hepatopancreas (Donker et al., 1990; Hopkin, 1990; Hames and Hopkin, 1991; Farkas et al., 1996; Drobne, 1997; Hornung et al., 1998b), and protocols for toxicity testing are available (Hornung et al., 1998a; Hornung et al., 1998b) although no standard methods have been developed for these organisms (i.e. ISO, ASTM, OECD).

The most widely used toxicological endpoints in isopod testing are survival (e.g. Drobne, 1997; Jansch *et al.*, 2005), growth and food consumption processes (e.g. Loureiro *et al.*, 2006), and reproduction (e.g. Vink and Kurniawati, 1996; Hornung *et al.*, 1997). The use of reproduction patterns as toxic responses is sometimes not convenient because of the long duration required for the test. Reproduction is also difficult to assess because after mating, females may retain the sperm for a long period of time before egg fertilization (Vink and Kurniawati, 1996; Drobne, 1997). On the other hand using reproduction as a response endpoint to test sublethal effects of chemicals has the advantage of not killing the animals during the procedure. The effects of chemicals to reproduction traits are crucial to understand and transpose those effects to higher levels of organization. The impairment of reproductive processes will be crucial for the population stability/growth and therefore isopods' role in decomposition processes and cycling of nutrients will be also affected.

Previous dietary studies on the assimilation of Cd in the terrestrial isopod *Porcellio dilatatus* (Chapter 2, Calhoa et al., 2006) indicated that the Cd speciation dictated the

assimilation efficiency (AE) of Cd in plant-isopod food chain. Among vascular plants, mechanisms of tolerance include the induction of metal-binding proteins such as phytochelatins and metallothioneins (Prasad, 1995) which are small proteins with a significant concentration of cysteine (30%) (Ndayibagira et al., 2007). Cysteine contains a sulphydryl group that accounts for the Cd-metallothionein induction due to Cd high affinity for sulphur ligands (Zalups and Ahmad, 2003; Roosens et al., 2005). The use of Cd-cysteinate in these studies provides an experimental device to explore the bioavailability of Cd that is complexed within biological tissues. Therefore Cd-cysteinate represents the most elementary form (species) of thiol-bound Cd in biological systems. In previous studies (Chapter 3) cadmium AE was lower among isopods provided with food with $Cd(Cys)_2$ demonstrating that the cysteine conjugate was less available when comparing with the isopods fed $Cd(NO_3)_2$.

In this study, the influence of cadmium speciation in the survival and reproductive effort of isopods was investigated. The main goal was to determine the toxicity effects of two species of Cd [$Cd(Cys)_2$ and $Cd(NO_3)_2$] on the survival and several reproductive parameters of the terrestrial isopod *Porcellio dilatatus* during a long term exposure period. The sublethal toxicity of dietary species of Cd to terrestrial isopods was measured as the time to reach pregnancy (as an indication of fertilization ability), pregnancy duration, pregnancy and/or inconclusive pregnancy, number of juveniles per female and juvenile weight.

5.2. Materials and Methods

5.2.1. Test organisms and culture conditions

Isopods were selected from laboratory cultures of *P. dilatatus* that have been maintained for more than 3 years and that were derived from individuals collected in a secondary coastal dune system in central Portugal. They were maintained on a substrate of sand in plastic containers at 20 ºC with a 16:8 h (light:dark) photoperiod. Alder leaves oven-dried were provided *ad libidum* as a food source (Caseiro et al., 2000; Kautz et al., 2000) and distilled water was added to maintain moisture.

5.2.2. Lettuce and gelatine substrate

A mixture of lettuce leaves and gelatine was selected as a suitable food substrate to be used as the exposure route (Mann et al., 2005; Monteiro et al., 2008). Non-contaminated

leaves of *Lactuca sativa* were reduced to powder using a mortar and pestle, and were mixed with a gelatine solution prepared from 2.5 g gelatine powder (VWR Prolabo, Fontenay Sous Bois, France) with 12.5 ml deionised water (Milli-Q®), and then mixed by vortexing (Wallace and Lopez, 1996). Small portions of the mixture (gelatine discs) weighing approximately 9 mg (dry wt) were made and were pipetted onto Parafilm® (Pechiney Plastic Packaging, Menasha, WI, USA). These discs were stored frozen at -20 ºC until required (Wallace and Lopez, 1997).

Three treatments (diets) were established for this long term exposure test to evaluate the toxicity of metal speciation to the terrestrial isopod *P. dilatatus*.

- $Cd(Cys)_2$ contaminated food - gelatine contaminated with Cd-cysteinate mixed with non-contaminated leaves of *L. sativa*;
- $Cd(NO_3)_2$ contaminated food - gelatine contaminated with Cd nitrate mixed with non-contaminated leaves of *L. sativa*.
- Control food - gelatine mixed with non-contaminated leaves of *L. sativa*.

5.2.3. Experimental setup

A total of 50 non-gravid females were selected and separated into a test box for one month, to guarantee that they were not pregnant when the test started. Tests boxes were made of polyethylene terephthalate (PET) (∅ 85 mm x 43 mm; Termoformagen, Leiria, Portugal) containing in the bottom a thin layer of sand to provide the same conditions as in the culture boxes. After this month a total of 30 males were also selected. At this stage (T0) 30 females and 30 males were exposed individually in test boxes for a period of 28 d (T1), and exclusively fed on gelatine discs according to treatment, to guarantee Cd assimilation. Hereafter this period will be named individual exposure test.

Gelatine discs were previously assayed for Cd radiospectrometry before being fed to isopods, and contained 335±29 µg Cd/g dry wt for gelatine with $Cd(NO_3)_2$ and 744±108 µg Cd/g dry wt (mean ± standard error) for gelatine with $Cd(Cys)_2$. Food was replaced every week, to prevent consumption of disks that had become inoculated with fungi, because fungi growth may alter Cd bioavailability.

After this 28 d period of individual exposure, one male and one female were paired randomly per box for mating, using 10 replicates per treatment. Hereafter this test period will be referred as reproduction test. Female reproductive cycle and survival was followed

for 54 days (T2), being monitored 3 times a week, and when pregnancy was observed females were moved into a new box alone until they gave birth. The percentage of females that successfully reached pregnancy (i.e. successful egg fertilization), the time until pregnancy, duration of pregnancy and the percentage of inconclusive pregnancies (females that successfully reached pregnancy but were unable to carry out in to the end) were recorded. The number of juveniles born per female and their individual weight were also registered. Isopods were assayed for Cd body burden by radiospectrometry, and metal contents were used to calculate their assimilation of Cd.

5.2.4. Statistical analysis

All data were checked for normality and homoscedascity using SigmaStat (version 3.01, SPSS, Chicago, IL, USA). Statistical analysis was carried out , for a significance level of 0.05, by t-tests or one-way analysis of variance (ANOVA) with Tukey test multiple comparison and with Dunnett´s multiple comparison of means to determine differences relatively to control treatment. Whenever possible data that was not normally distributed or whose equal variance testing failed were transformed.

The lethal time of 50% (LT_{50}) was calculated with the Probit method using the MiniTab software (Minitab, 2000).

5.3. Results

5.3.1. Survival

Survival is registered in Figure 5.1. During the individual exposure period no mortality was observed.

During the 82 days of exposure, there was no mortality on control females while 20% of the males died during the test. In the $Cd(Cys)_2$ treatment 70% of females and 90% of males have died at the end of the test. As for the $Cd(NO_3)_2$ treatment mortality was of 10% and 70%, respectively for females and males, being the differences between the gender extremely high. LT_{50} values for $Cd(Cys)_2$ exposures differed between females and males: 65.85 days (63.97-67.89 for 95%CL) and 58.35 days (56.80-59.89 for 95%CL), respectively. For the $Cd(NO_3)_2$ exposure only data from male survival gave a LT_{50} value of 70.36 days (68.44-72.57 for 95%CL). Females exposed to $Cd(NO_3)_2$ showed a low mortality rate of 10% (figure 5.1).

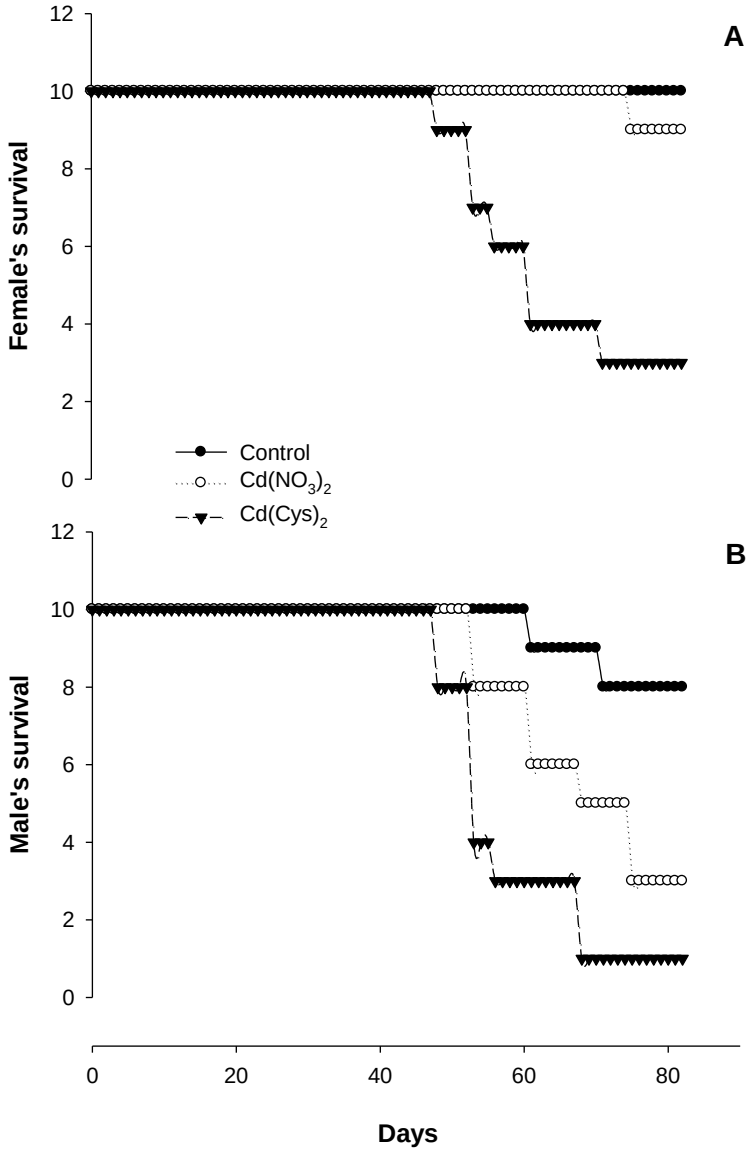

Figure 5.1. Number of females (A) and males (B) of *Porcellio dilatatus* that survived during the 82 days of the test.

5.3.2. Cd assimilation in isopods

Isopods fed with $Cd(NO_3)_2$ and $Cd(Cys)_2$ had equivalent rates of assimilation (6,4±1,9 and 7,8±1,7ng Cd/mg animal, (mean ± standard error, respectively) after the individual exposure period of 28 days (T1) (Figure 5.2). In the final of the experiment (T2) isopods fed with $Cd(NO_3)_2$ assimilated much more Cd (17,9ng Cd/mg animal) than those from the $Cd(Cys)_2$ treatment that only assimilate 9,9ng Cd/mg animal.

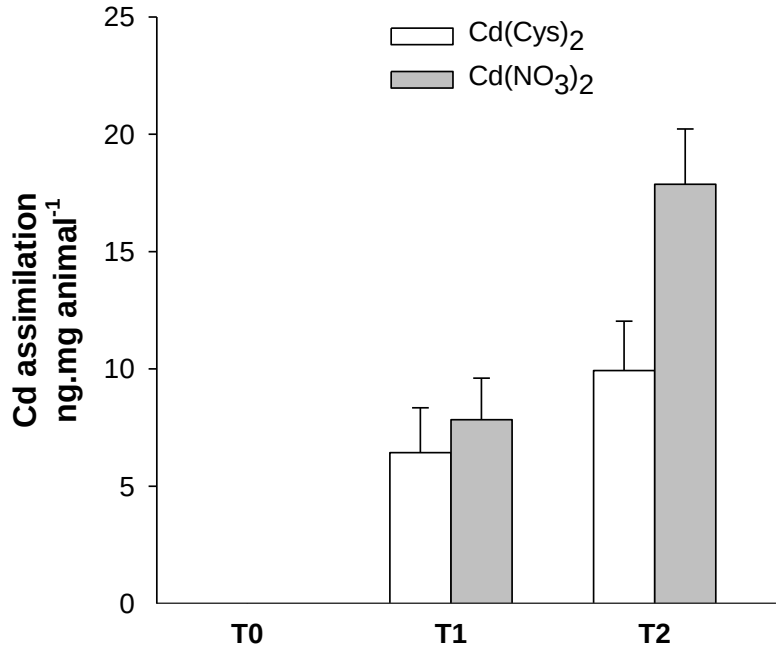

Figure 5.2. Cadmium assimilation by *Porcellio dilatatus* before the experiment starts (T0), after 28 days of individual exposure (T1) and at the final of the experiment (82 days of exposure) (T2). Grey bars represent the assimilation in isopods fed with $Cd(NO_3)_2$ and the white bars represent the assimilation in isopods fed with $Cd(Cys)_2$. Vertical error bars represent the standard error of the mean (n=4 to $Cd(Cys)_2$ and n=12 to $Cd(NO_3)_2$).

5.3.3. Time to reach pregnancy, pregnancy duration and abortions

During the individual exposure test, plus the 54 days of the reproduction test, all animals lost weight, with the exception of the only male that survived in the $Cd(Cys)_2$ treatment, that showed a growth rate of 14.22%. There were no significant differences between treatments and within sexes (female data after exponential transformation, P=0.117; males, P=0.376).

As shown in Figure 5.3, the average time after mating at which isopods showed the first signs of pregnancy in control isopods were of 19±2 days (mean±st. error). This interval was lower for isopods fed with $Cd(NO_3)_2$ (12±1 days) and even lower for $Cd(Cys)_2$ (10±2 days). Statistical differences were found between the control and the two Cd treatments ($p<0.05$). Average pregnancy duration was of 21±1 days for the control, and

decreased in $Cd(NO_3)_2$ treatment (14±3 days) whereas for the $Cd(Cys)_2$ treatment no females were able to deliver mancae.

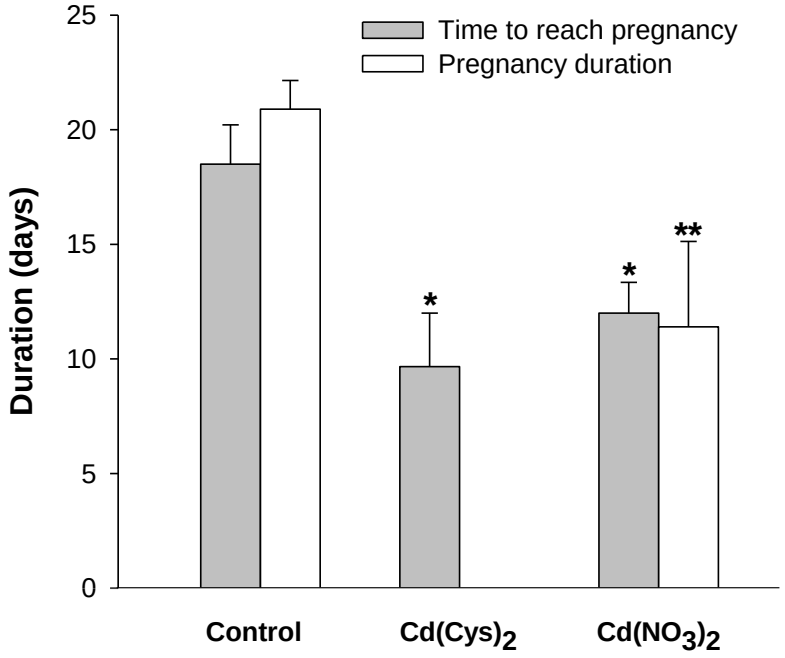

Figure 5.3. Number of days until first signs of pregnancy were detected (grey bars), and number of days between first signs of pregnancy and release of manca (white bars) of *Porcellio dilatatus* fed with $Cd(Cys)_2$ and $Cd(NO_3)_2$ gelatine and lettuce discs. *n* ranges from 3 to 10. Vertical error bars represent the standard error of the mean. "*" indicates a significant difference (P<0.05) from the control (ANOVA, Dunnett´s test); "" indicates a significant difference (P<0.05) between the treatments (t-test).**

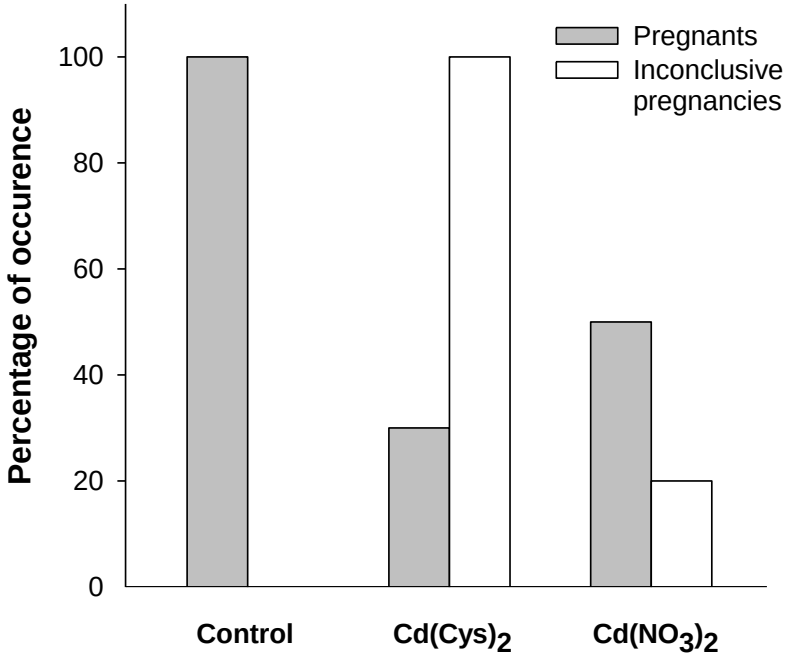

Figure 5.4. Percentage of successful females achieving pregnancy (grey bars) and percentage of female inconclusive pregnancies (white bars) of *Porcellio dilatatus* feed with $Cd(Cys)_2$ and $Cd(NO_3)_2$ gelatine and lettuce discs.

In the control all the females successfully reached pregnancy within the test period (Figure 5.4). Only 30% of the females fed with $Cd(Cys)_2$ become pregnant but all these females did not produce any manca (two of them died and the one that survived did not delivered any manca due to inconclusive pregnancy). In the $Cd(NO_3)_2$ treatment only half of the females successfully reached pregnancy, but only 80% of these were able to carry it till the end.

5.3.4. Number of juveniles and individual juvenile weight

Significant differences were found between the number of juveniles in the control and the Cd treatments ($P<0.001$), but differences between the $Cd(Cys)_2$ and the $Cd(NO_3)_2$ were not statistically significant (Figure 5.5). In the control, the average number of mancae delivered per female was 21±2. In the $Cd(Cys)_2$ none of the females were able to carry the pregnancy to the end so no juveniles were delivered. In the $Cd(NO_3)_2$ the number of mancae delivered per female was 7±2.

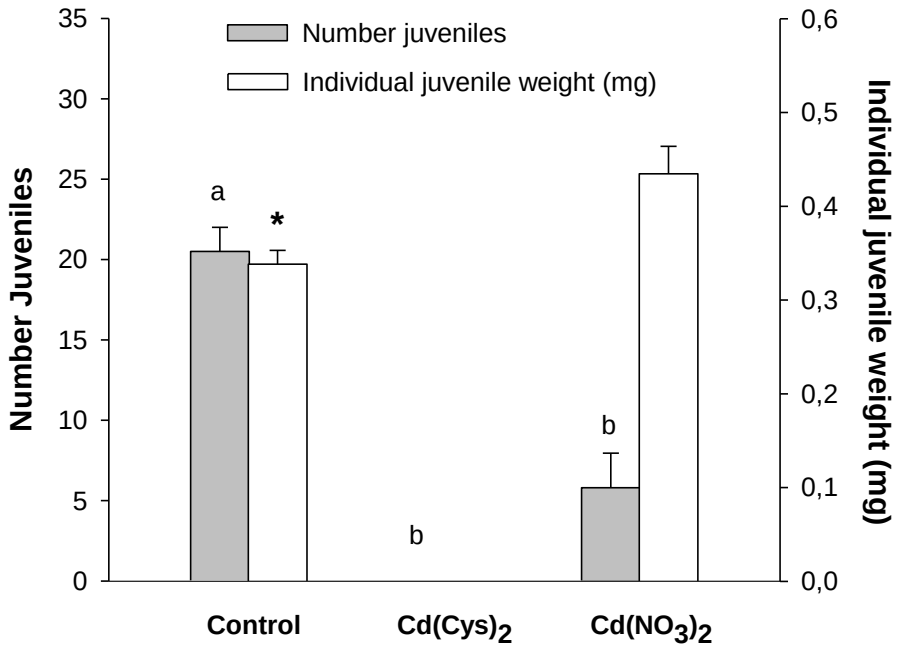

Figure 5.5. Number of juveniles hatching per pregnant female (grey bars), and individual manca weight (white bars) of *Porcellio dilatatus* fed with $Cd(Cys)_2$ and $Cd(NO_3)_2$ gelatine and lettuce discs. Vertical error bars represent the standard error of the mean. a and b indicates a significant difference (P<0.001) between number of juveniles produced in the treatments (ANOVA, Tukey test); asterisk indicates a significant difference (p=0.007) between juvenile individual weight (t-test).

The total juvenile weight was significantly different between the control and $Cd(NO_3)_2$ treatment (P<0.001). In the control there were more juveniles delivered, and therefore the total weight (6.8±0,31 mg) was higher than in the $Cd(NO_3)_2$ (3.3±0,96mg). But associated with the decreasing number of mancae per female in $Cd(NO_3)_2$, the individual manca weight increased. There were significant differences between individual weight of the control juveniles and those from the $Cd(NO_3)_2$ treatment (p=0.007). In the control more juveniles were delivered but the individual weight was lower (0.338±0.015mg) than in the $Cd(NO_3)_2$ that produced less juveniles but with higher individual weight (0.435±0.029mg).

5.4. Discussion

Metal contamination is one of the most important factor responsible for the change on life-history patterns in isopods because it acts as a selection pressure, being reproduction a highly sensitive life-history parameter (Hornung et al., 1998b). Life-history theory predicts that habitat disturbances will increase mortality, select for early reproduction and increase reproductive effort (Donker et al., 1993).

From the survival data, there was an unexpected difference between sexes in both exposures. In several works no differentiation between sexes is considered due to the expected similarity on pattern behaviour and survival of terrestrial isopods (Jansch et al., 2005; Calhoa et al., 2006; Loureiro et al., 2009). The inexistence of differences between sexes in isopods was described in the study where the aquatic isopod *Idotea baltica* was exposed to zinc, copper and lead and no differences were observed between males and females LT_{50} values (Bat et al., 1999). In the present work males exposed to $Cd(Cys)_2$ died earlier than those exposed to $Cd(NO_3)_2$, showing higher acute toxicity.

Influences of chemical speciation on the reproduction systems of terrestrial isopods, with consequences at the population level, are expected but have not been confirmed until now. Isopods fed with $Cd(NO_3)_2$ and $Cd(Cys)_2$ had equivalent Cd assimilation rates (6.4 and 7.8ng Cd/mg animal, respectively) after the 28 days (T1) of individual exposure. A higher concentration of Cd in $Cd(Cys)_2$ gelatine discs was provided to try to equal metal assimilation by isopods fed with both Cd species, because in previous experiments (Chapter 3) isopods fed with $Cd(Cys)_2$ assimilated less Cd than those exposed to $Cd(NO_3)_2$ contaminated food. It was important to have similar assimilation rates for a reproduction test to compare what happens in terms of toxicity for both Cd species. At the end of the experiment (T2) isopods fed with $Cd(NO_3)_2$ contaminated food assimilated much more Cd (17.9ng Cd/mg animal) than those from $Cd(Cys)_2$, that only assimilated 9.9ng Cd/mg animal.

In a previous study (Chapter 4) it was also shown that isopods fed with $Cd(Cys)_2$ gelatine discs had a higher storage level of Cd in the metal-sensitive fractions (MSF). It is hypothesized that Cd in organelles and in heat denatured proteins (HDP) can be considered potentially vulnerable fractions, and in the present study it is demonstrated how it affects isopods in terms of toxicity. The $Cd(Cys)_2$ treatment had the highest mortality probably due to higher availability of the ingested Cd that impaired physiological processes. Isopods fed with $Cd(NO_3)_2$ stored Cd in metal-rich granules (MRG) as a detoxification strategy, so they were more efficient at detoxifying Cd which may had lead

to increased metal body burdens although being less toxic to the isopod. In this way, Cd in granules is not available for the physiological processes and become non toxic. This could be also related to metal tolerance and resistance that may be attributed to the ability of isopods to compartmentalize Cd in the hepatopancreas, which acts as a detoxification mechanism and contributes to tolerance to high cadmium levels. MRG has been found in intestinal cells of many invertebrates (Vijver et al., 2006), including isopods (Dallinger and Prosi, 1988), where it is known that Cd in the hepatopancreas is mainly present in the S (small) cells, which consist of granules.

Jones and Hopkin (1996) showed that terrestrial isopods (*Porcellio scaber* and *Oniscus asellus)* from metal polluted sites had higher reproductive investment, suggesting that they were able to redirect resources from other functions, like growth, to meet the physiological costs of metal detoxification. Donker et al. (1993) also showed that isopods from a zinc smelter started to reproduce earlier and that metal contamination decreased the adult survival, but the reproductive effort was higher when compared to reference site isopods. In our experiment, isopods were previously exposed to Cd for 28 days, to evaluate the effects of Cd on the egg fertilization phase.

During the 28 days of the individual exposure test plus the 54 days of the reproduction test all animals lost weight, probably due to the long duration of the test, in involving a long period of stress. Growth inhibition among isopods is a commonly reported consequence of Cd exposure (Odendaal and Reinecke, 2004), and in this study may reflect the increased cost of detoxification of Cd^{2+} (Zidar *et al.*, 2003). Considering the high rates of mortality observed in this study, although isopods exposed to $Cd(NO_3)_2$ assimilated more Cd, higher female mortalities were observed in the $Cd(Cys)_2$ treatments, showing that $Cd(Cys)_2$ induced higher acute toxicity.

In the control all females become pregnant and delivered mancae within the 54 days of the reproduction test. These values are much higher than the few reports available with other isopod species but are in accordance with Lemos et al. (2009) that reported the same percentage of successfully pregnancy (100%) for *Porcellio scaber* females. Van Brummelen et al. (1996) reported a 57% failure of pregnancy in *Oniscus asellus*, Faber and Heijmans (1995) reported a 49% failure in *Trachelipus rathkei*, and Hornung and Warburg (1994) reported a 47% failure in *Porcellio ficulneus*.

Control females showed the first signs of pregnancy later than the Cd treatments, which is in accordance with life-history theory that predicts a selection for early reproduction and increase reproductive effort when these organisms are stressed (Donker

et al., 1993). As mentioned above, metal exposure is responsible for changes on life-history, acting as a selection pressure, being reproduction a highly sensitive life-history parameter (Hornung et al., 1998b). As observed in this study and also reported by Donker et al. (Donker et al., 1993), stress increased mortality, and early reproduction, with an additional increase in reproductive effort.

Pregnancy duration was higher for the control treatments and decreased in the $Cd(NO_3)_2$ treatment, where juveniles were released, on average, seven days earlier. As Cd induced a decrease of the brood period, females exposed to $Cd(NO_3)_2$ had a reduced length of time to provision for their developing young. A similar observation was observed in females of *Armadillidium vulgare* exposed to predatory ants (Castillo and Kight, 2005) and females of *Porcellio leavis* under physical (five minutes of continuous locomotion daily throughout the brooding period) stress (Kight and Nevo, 2004) that exhibited significantly shorter brooding periods than controls, releasing juveniles almost 48 hours earlier. Parental care by female isopods is a behavioural strategy that contributes to increase fitness of progeny but is energetically costly (Lardies et al., 2004). Terrestrial isopods have the most extensive parental care since they carry the eggs and juveniles in a ventral marsupium, that is provisioned with fluid from the mother and allows early development to take place independently of an external water source (Lardies et al., 2004). The equilibrium between energy losses with reproduction/ maternal care and those from detoxification processes due to Cd exposure might justify the decrease in the number of days of isopod pregnancy, to shift energy for detoxification.

Terrestrial isopods' female reproductive cycle, i.e. ovarian maturation and embryogenesis, is a synchronous event with the moult cycle. Ecdysteroids are crustacean's moulting hormones, involved in controlling the reproduction process and embryogenesis (Vafopoulou and Steel, 1995; Subramoniam, 2000; Lemos et al., 2009). Metals interfere with egg production binding to enzymes involved with vitellogenesis (Hook and Fisher, 2002). Thus any impact on the moulting process has negative consequences at the reproductive success. Hornung and Warburg (1994) observed for the first time the oosorption in an ovary of an oniscid isopod (*P. ficulneus*) and Farkas et al. (1996) showed that *P. scaber* under stressful conditions increased disruption of oocytes within the ovarium during vitellogenesis (oosorption) resulting in fewer juveniles. The isopod marsupium physically limits the area available for egg attachment, and abnormal egg growth may lead to overcrowding and consequent egg loss or reabsorption, following-on lower manca numbers (Lardies et al., 2004). This phenomenon may had occurred in the present experiment with the $Cd(NO_3)_2$ treatment. The oocyte resorption

takes place when the albumin is stopped from entering the oocyte and no vitellogenin synthesis takes place. This process leaves no visible traces after the reasorption has been completed. In the $Cd(Cys)_2$ none of the females were able to carry the pregnancy to the end (most of them because animals died before breeding) and the only that survived did not deliver any manca, due to inconclusive pregnancy.

In the control the average number of mancae delivered per female was of 21±2. In the $Cd(NO_3)_2$ treatment the number of mancae delivered per female decreased when compared to the control, but the individual weight increased. Probably upon stress exposure isopods invest in quality rather than in quantity of juveniles to produce a higher quality and fit offspring. Among crustaceans, fecundity has been demonstrated to be particularly sensitive to dietary metal exposure (Hook and Fisher, 2001; Mann and Hyne, 2008; Mann *et al.*, 2009). Hence we suggest that females from $Cd(NO_3)_2$ balanced the benefits of having smaller clutch decreasing the costs of providing parental care, which implies less energy for maintenance.

5.5. Conclusion

The results from the present study demonstrate that different species of Cd affect survival and reproduction of terrestrial isopods in different ways. In fact speciation had already been shown to affect the assimilation efficiency and compartmentalization of Cd in isopods (Chapters 2 and 3). The compartmentalization results were of particular interest because they showed that Cd accumulates differently depending on the form it is provided in food. Isopods fed with $Cd(Cys)_2$ gelatine discs had a higher storage level of Cd in the metal-sensitive fractions (organelles+ heat denatured proteins) that are responsible for toxicity. As for isopods fed with $Cd(NO_3)_2$ they stored Cd in metal-rich granules as a detoxification strategy so they were more efficient at detoxifying Cd. Thus it was expected that the effects of $Cd(Cys)_2$ would be more toxic than the effects of $Cd(NO_3)_2$. In agreement with this, survival and reproduction were also affected differently depending on Cd speciation. There was a difference between survival rates of $Cd(Cys)_2$ exposed males and females and an higher acute toxicity was also observed when compared to males exposed to $Cd(NO_3)_2$. In both treatments a reduction of pregnancies and pregnancy duration was observed but in the case of $Cd(Cys)_2$ all females had an inconclusive pregnancy and therefore no juveniles were delivered. Although in $Cd(NO_3)_2$ the number of juveniles per female was lower than in the control, juvenile weights were higher. As far as

we are aware, the present study is the first one demonstrating that metal speciation affects reproduction.

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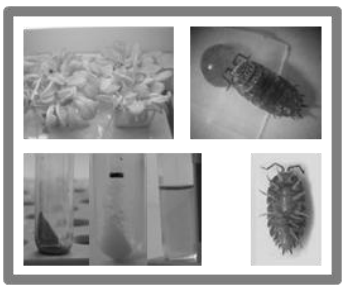
CHAPTER 6



Chapter 6. General Discussion

The focus of this research was to investigate the influence of cadmium speciation in its bioavailability to the isopod *Porcellio dilatatus* (Figure 6.1). We wanted to know if Cd speciation had consequences in Cd assimilation (Experiment 1 and 2), in the way metal is distributed internally within the organism (Experiment 3), and how survival and reproduction were affected in terrestrial isopods (Experiment 4) (Figure 6.1).

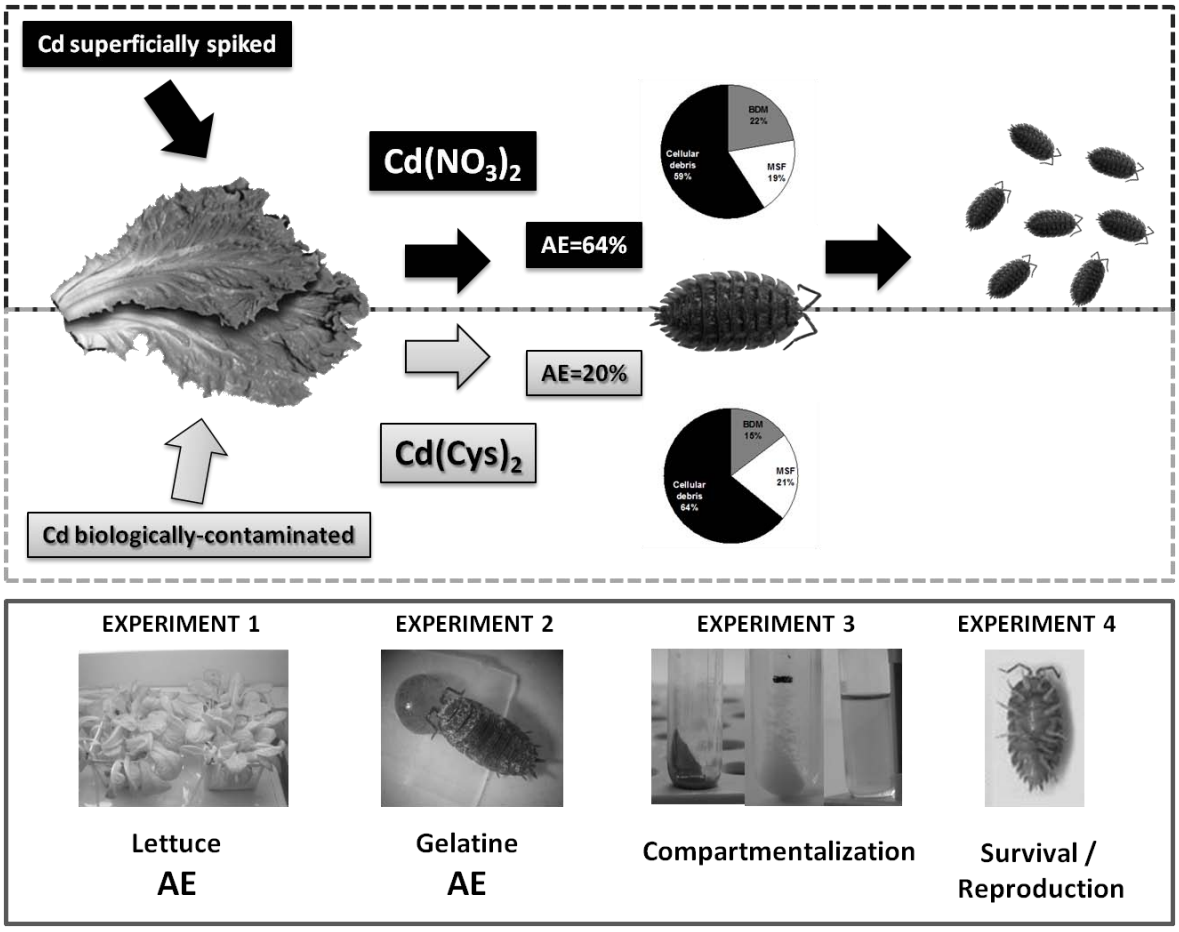


Figure 6.1. Schematic sequence of the experimental set-up.

Bioavailability of the metals when presented through doping regimes may differ from the bioavailability of metals in nature, because over time metals become biologically compartmentalized and complexed by organic molecules. Therefore, the first step of this study was to evaluate the Assimilation Efficiency (AE) of Cd in isopods provided with food (lettuce) superficially amended with $Cd(NO_3)_2$ and provided with lettuce grown in Cd-contaminated media (Chapter 2). One of the *a priori* assumptions for these trials was that Cd incorporated biologically into the lettuce must exist predominantly as a Cd-*S*-conjugate or Cd-protein complex (Mann et al., 2005). This assumption was born out by a subsequent sub-cellular fractionation study (Monteiro et al., 2008) that indicated that 22.4% of Cd was bound to the heat stable protein fraction (Metallothionein-like proteins). The results from our experiments showed that the AE of Cd was greater among isopods that were fed the simple salt (71%, SE=7%), than among isopods feeding on biologically contaminated lettuce (52%, SE=5%), hence demonstrating that speciation of Cd is likely to influence the rate of Cd assimilation and accumulation in a laboratory test, unlike the findings from Harrison and Curtis (1992) where the AE was higher in the case of Cd accumulated by a live food source.

In the following trial we used Cd-cysteinate to provide an experimental device to explore the bioavailability of Cd that is complexed within biological tissues (Chapter 3). Cysteine is the primary source of sulfydryl ligands in metal-binding proteins such as Metallothionein (MTs). Food provided in treatments consisted of gelatine contaminated with Cd cysteinate ($Cd(Cys)_2$) and $Cd(NO_3)_2$. In this dietary study as well as in the study performed with contaminated lettuce, growth was poor. Growth inhibition among isopods is a commonly reported consequence of Cd exposure (Odendaal and Reinecke, 2004), and the fact that food assimilation in the $Cd(NO_3)_2$ treatment group was similar to controls indicated that the poor growth indices were not simply a reflection of an avoidance behaviour (Odendaal and Reinecke, 1999), but rather may had reflected the increased cost of detoxification of Cd^{2+}. The AE of $Cd(Cys)_2$ was relatively low (20%) when compared to $Cd(NO_3)_2$, and much lower than the Cd-AE in the biologically-contaminated lettuce group (~50%) from the first experiment, which confirms the relatively low bioavailability of Cd associated with Cd-*S*-conjugates (Harrison and Curtis, 1992; Andersen et al., 2004; Mann et al., 2006; Monteiro et al., 2008). Monteiro et al. (2008) examined the subcellular distribution of Cd in lettuce following hydroponic contamination, and demonstrated that only a small proportion of metal (22.4%) was bound to a subcellular fraction (HSP) synonymous with phytochelatins or MT-like proteins. In the same study, isopods were provided with isolated subcellular fractions, and similarly

demonstrated that the Cd in the fraction containing Cd-*S*-conjugates (HSP) had a low AE (22.8%), which was close to the AE for Cd in $Cd(Cys)_2$.from the second experiment. These data suggest that the isopods' ability to assimilate $Cd(Cys)_2$ was the same as their ability to assimilate Cd-MT.

Chapter 4 examines the subcellular distribution of Cd when presented to isopods as the different Cd species – $Cd(Cys)_2$ and $Cd(NO_3)_2$ –, and the hypothetical difference in toxicity. The compartmentalization of metal as a subcellular compartment containing metal-sensitive fractions (MSF) is related to toxicity and is the metal considered metabolically available (Bechard et al., 2008). The biologically detoxified metal (BDM) compartment is related to metal-detoxifying capacity of an organism and potential tolerance (Wallace et al., 1998; Goto and Wallace, 2007), providing a more complete understanding of potential mechanisms of toxicity (Wallace et al., 2003). The cellular debris is the only subcellular fraction that was not included in the compartmental analysis and includes tissue fragments, cell membranes and other cellular components of unknown consequence in terms of function. This compartmentalization experiment revealed that the cellular debris had the highest subcellular Cd distribution (59-64%) independently of the species of Cd, which was in good agreement with results obtained by Monteiro et al. (2008) for Cd subcellular distribution of *L. sativa* deployed to the isopod *P. dilatatus*. On cellular sequestration there are two major strategies of detoxification. One involves the binding of metals to heat-stable proteins (HSP) and the second one involves the formation of metal-rich granules (MRG). The role of MT in Cd binding (i.e. percentage of Cd bound to HSP) compared to the other subcellular fractions appeared to be lower in our study when compared to other investigations with organisms exposed to Cd via food (Giguere et al., 2006; Monteiro et al., 2008). In both tested species of Cd, the HSP fraction did not appear to play an important role in detoxification processes for Cd as it would be expected (2,9% in $Cd(Cys)_2$ and 3,3% in $Cd(NO_3)_2$). The second detoxification strategy in invertebrates is metal storage in MRG. *P. dilatatus* fed with $Cd(NO_3)_2$ accounted for 19% in MRG, whereas only 11,8% of the total Cd was found in MRG of *P. dilatatus* fed with $Cd(Cys)_2$. MRG has been found in intestinal cells of many invertebrates (Vijver et al., 2006), including isopods (Dallinger and Prosi, 1988), where is known that Cd that enters in the hepatopancreas is mainly present in the S (small) cells, which consist of granules. If sequestration as HSP and MRG (BDM) is considered a mode of detoxification, we can suggest that isopods fed with $Cd(NO_3)_2$ were more efficient at detoxifying Cd (22%) than when fed Cd(Cys) (15%), which can lead to increased metal body burdens although being less toxic to the isopod. This could be also related to metal tolerance and resistance,

being such subcellular compartmentalization approach important to interpret differences in toxicity. In sum, this study showed that total tissue burdens in prey may not be directly related to metal transfer to predators and the subcellular partitioning results were more useful when individual fractions were grouped into compartments MSF and BDM. Such findings demonstrate that the variability observed in metal partitioning can be useful in explaining toxicity. Moreover herewith it was shown that the subcellular distribution of Cd in isopods can be modified by metal speciation and subcellular fractionation of metal-binding in tissues, clarifying the mechanisms for metal toxicity and how the organisms detoxify metals. The results highlighted that a change in the speciation of Cd may have had a direct impact in the Cd subcellular distribution hence affecting the trophic transfer.

In a final set of experiments, detailed in Chapter 5, the differences in terms of survival and reproduction in isopods exposed to $Cd(Cys)_2$ and $Cd(NO_3)_2$ were assessed. From the survival data, there was an unexpected difference between sexes in both exposures. Males exposed to $Cd(Cys)_2$ died earlier than those exposed to $Cd(NO_3)_2$, showing higher acute toxicity. In the control all females become pregnant and delivered mancae within the 54 days of the reproduction test. Control females showed the first signs of pregnancy later than the Cd treatments which is in accordance with life-history theory that predicts a selection for early reproduction and increase reproductive effort when stressed (Donker et al., 1993). Pregnancy duration was higher for the control treatments and decreased in $Cd(NO_3)_2$ treatment where juveniles were released seven days earlier. As Cd induced a decrease of the brood period, females exposed to $Cd(NO_3)_2$ had a reduced length of time to provision for their developing young. Terrestrial isopods have the most extensive parental care since they carry the eggs and juveniles in a ventral marsupium, that is provisioned with fluid from the mother and allows early development to take place independently of an external water source (Lardies et al., 2004). The equilibrium between energy losses with reproduction/ maternal care and those from detoxification processes due to Cd exposure might justify the decrease in the number of days of isopod pregnancy, to shift energy for detoxification. In the $Cd(NO_3)_2$ the number of mancae delivered per female decreased when compared to the control (21±2), but the individual weight increased. Probably upon stress exposure isopods invested in quality rather than in quantity of juveniles to produce a higher quality and fit offspring. Among crustaceans, fecundity has been demonstrated to be particularly sensitive to dietary metal exposure (Hook and Fisher, 2001; Mann and Hyne, 2008; Mann *et al.*, 2009). Hence upon the obtained results we suggested that females from $Cd(NO_3)_2$ balanced the benefits of having smaller clutch decreasing the costs of providing parental care, which implies less

energy for maintenance. At the end of the experiment isopods fed with $Cd(NO_3)_2$ contaminated food assimilated much more Cd (17.9ng Cd/mg animal) than those from $Cd(Cys)_2$ that only assimilated 9.9ng Cd/mg animal. In Chapter 4 it was also shown that isopods fed with $Cd(Cys)_2$ gelatine discs had a higher storage level of Cd in the metal-sensitive fractions (MSF). It was then hypothesized that Cd in organelles and in heat denatured proteins (HDP) could be considered potentially vulnerable fractions and in chapter 5 it was demonstrated how it affects isopods in terms of toxicity. The $Cd(Cys)_2$ treatment had the highest mortality probably due to higher availability of the ingested Cd that impaired physiological processes. Isopods fed with $Cd(NO_3)_2$ stored Cd in metal-rich granules (MRG) as a detoxification strategy so they were more efficient at detoxifying Cd which may had lead to increased metal body burdens although being less toxic to the isopod. In this way, Cd in granules was not available for the physiological processes and become non toxic. This could also be related to metal tolerance and resistance that may be attributed to the ability of isopods to compartmentalize Cd in the hepatopancreas, which acts as a detoxification mechanism and contributes to tolerance to high cadmium levels.

As a final remark it can be suggested that future studies examining the trophic movement of metals in food chains should consider this kind of approach, where different flows within a trophic chain are expected depending on metal speciation.

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